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NITROGEN SOURCES AND CYCLING IN NATURAL WATERS

Patrick L. Brezonik

Florida University

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16. Abstract

Sources of nitrogen were reviewed to determine their significance in lake nitrogen budgets. Nutrients in rainfall were evaluated and found to be significant. Nitrogen and phosphorus budgets were calculated for 55 Florida lakes and critical loading rates established by comparing calculated budgets with data on trophic state.

Nitrogen fixation by Cyanophyceae was studied in detail in two eutrophic Florida lakes for one year. Also a survey of fixation in Florida lakes was conducted and fixation found only in eutrophic lakes. Bacterial fixation was found to contribute significant nitrogen to the anoxic hypolimnon of a small stratified lake. Nitrogen fixation was found in both lacustrine and estuarine sediments.

Sediments of 55 lakes were characterized chemically and results suggest that such sediment may act as an ammonia buffer, sorbing ammonia at high concentrations and releasing it to ammonia depleted water. Estuarine sediment sorbed ammonia strongly but failed to release it to overlying water.

The acetylene reduction assay for nitrogen fixation was evaluated. Interferences in automated nutrient determinations due to organic color were studied and simple color correction found for nitrite, nitrate and orthophosphate but not ammonia as determined by the indophenol method. Amino acids also interfered with the ammonia analysis. ~~(Keirn-Florida)~~ (EPA)

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NITROGEN SOURCES AND CYCLING IN NATURAL WATERS

By

Patrick L. Brezonik
University of Florida
Department of Environmental Engineering Sciences
Gainesville, Florida 32601

Research Grant No. 16010-DCK

Project Officer

Dr. Charles F. Powers
National Eutrophication Research Laboratory
Pacific Northwest Environmental Research Laboratory
Corvallis, Oregon 97330

Prepared for

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Abstract

Sources of nitrogen were reviewed to determine their significance in lacustrine budgets. Nutrients in rainfall were found significant although their variability obviates precise conclusions. Using literature values for nutrient export from various land uses, nutrient budgets were calculated for 55 Florida lakes. Critical N and P loading rates (above which eutrophication is likely) were estimated from the calculated budgets and lake trophic conditions.

Algal fixation in two eutrophic Florida lakes was studied in detail; the total annual N fixed and factors affecting the occurrence of fixation were evaluated. A survey of fixation in 55 Florida lakes showed significant fixation only in eutrophic lakes. Bacterial fixation in the anoxic hypolimnion of a small lake contributed substantial nitrogen to the lake, and N fixing activity was found in both estuarine and lacustrine sediments. The acetylene reduction assay for N fixation was evaluated; short incubations were found essential. Reduction was light dependent and N_2 acted as a competitive inhibitor.

A preliminary experiment suggested that lacustrine sediments act as ammonia buffers; estuarine sediment sorbed ammonia strongly with little tendency to release ammonia to the water. Interferences from high organic color were evaluated for automated inorganic N and P analytical methods. Various amino acids were also shown to interfere with the indophenol ammonia procedure.

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SECTION I

CONCLUSIONS

1. Rainfall contributes substantial amounts of nitrogen to lakes. The great variability in concentrations obviates simple assessment of the significance of this source, and experimental evaluation for each lake seems necessary for accurate results. Nutrient concentrations decline rapidly with length of rainfall, indicating the role of precipitation as an atmospheric cleansing agent.

2. Using literature values for nutrient export from various land uses and easily evaluated watershed characteristics, one can compute nutrient budgets for lakes that, if not totally accurate, are nevertheless meaningful and useful in assessing the eutrophication potential of a lake. This approach, with refined export values would seem especially useful for small but still recreationally important lakes, where the time and expense of experimental nutrient budget evaluation cannot be justified.

3. Nitrogen fixation by blue green algae in eutrophic surface waters is of doubtless ecological significance, but in terms of contributions to the total nitrogen budgets of lakes fixation probably represents a relatively minor source. Fixation is found only in moderately or highly eutrophic waters where it likely exascerbates the problem by supplying more nitrogen during times of relative depletion. However, if external nutrient supplies are decreased below critical (eutrophying) levels, fixation itself is unlikely to cause continued problems.

4. Bacterial fixation in anoxic lake waters is now an established fact, and in some cases it can supply significant amounts of nitrogen to a lake system. Fixation in sediments is also established, and heterotrophic bacteria have been identified as the responsible agents in the sediments studied here. The ecological significance of this phenomenon remains puzzling, but the occurrence of fixation suggests that nitrogen is relatively unavailable at least in some sediments.

5. Non-enzymatic chemical equilibria, in particular sorption reactions, play an important, perhaps dominant role in controlling ammonia interchange between sediments and water. Sorption of ammonia onto sediments is, at least in some cases, rapid and largely irreversible.

6. The acetylene reduction method is a useful, precise and simple method for assaying nitrogen fixation in environmental samples. Short incubations (approximately one hour) should be used for accurate rate determinations. Acetylene reduction by algae is strongly light dependent. Molecular nitrogen inhibits acetylene reduction, and a pattern of competitive inhibition is obtained from Lineweaver-Burk plots. This fact is useful in establishing the enzymatic nature of acetylene reduction in environmental samples. Atmospheric levels of N_2 decrease acetylene reduction rates by about 25-30 percent, and if only approximate results

are desired, removal of N_2 may not be necessary.

7. The interference of organic color in automated nitrite, nitrate and ortho phosphate analyses can be simply compensated for by the method of blank subtraction, but this cannot be done with the indophenol ammonia method. Organic color or some constituent associated with color (e.g. iron) causes positive interferences with this method which cannot be corrected by eliminating a key reagent and measuring the color "blank." Further, a variety of amino acids also react as ammonia in the indophenol method, in some cases giving greater responses than equivalent ammonia-N levels. This method should thus be used with caution, especially when free amino acids are suspected in a water sample. When high color is present, a alternate ammonia procedure would be preferable.

SECTION II

RECOMMENDATIONS

1. Various diffuse sources of nitrogen, such as agricultural runoff, sanitary landfill sites, and urban runoff, need much further study to assess their quantitative significance in lake nutrient budgets.

2. The extent of denitrification in wetlands and sediments needs to be evaluated to properly assess the importance of this reaction as a nitrogen sink for lakes.

3. The relationships between nitrogen-fixing blue-green algae and other bloom forming algae need further investigation in terms of the transfer of nitrogen from fixers to non-fixers.

4. The role and ecological significance of nitrogen fixing activities in lacustrine and estuarine sediments need further exploration.

5. The role of sediments as nitrogen reservoirs or sinks needs further clarification. Experiments involving laboratory model systems and *in situ* methods should be undertaken.

SECTION III

INTRODUCTION

Of the major nutrient cycles in natural waters, the nitrogen cycle is perhaps the most interesting, most complex and least understood from a quantitative point of view. The geocycle of nitrogen is largely a biochemical phenomenon; in natural waters it is nearly wholly so. As such, the nitrogen cycle, like the carbon and phosphorus cycles, is inextricably related to aquatic organic productivity. While many elements and compounds are required for biosynthesis, nitrogen and phosphorus have long been considered the prime limiting nutrients for primary production; recent evidence suggests carbon may also limit production in some situations. The great recent concern over cultural eutrophication has stimulated much new research into the chemistry and biochemistry of these nutrients in aquatic systems, into quantifying the sources and sinks of nutrients and into the dynamics of nutrient uptake and release. This report discusses these subjects with emphasis on the cycle of nitrogen in natural waters.

The nitrogen cycle in natural waters has been studied over a long period of time. Qualitative aspects of the cycle are well known, but quantitative studies are much less advanced. For example, in situ rates of nitrogen cycle reactions have only recently been determined in a few lakes using ^{15}N. Rates of ammonia release from lacustrine sediments are virtually unknown, and determination of the nitrogen contributions from various sources is still in a crude and elementary state. The significance of natural and uncontrollable cultural sources like urban and agricultural runoff must be placed in proper perspective with the one nutrient source most often cited in the popular press, i.e. domestic waste effluent. If uncontrollable sources are alone sufficient to effect eutrophy in a lake, then a priori decisions to limit waste effluent as a nutrient source may be highly costly to the taxpaper without the anticipated benefits in improved water quality. On the other hand, it may be found that reduction of nutrients in controllable sources may significantly improve or completely restore water quality. In any event, decisions to limit nutrient flux by constraining certain sources must arise from knowledge of the entire nitrogen budget rather than from ignorance or speculation. To understand the factors controlling the concentrations and temporal variations of nitrogen forms in natural waters requires two types of research efforts. The first involves quantitative studies of the sources and sinks comprising the nitrogen budget of a water body; the second involves determination of the internal dynamics or in situ turnover rates of the different nitrogen forms in the water body.

The overall objective of the project was to study the factors controlling the concentration and forms of nitrogen in natural waters. To this end the project consisted of two principal phases:

1) delineation of the relative importance of certain source and sink components in nitrogen budgets and;

2) investigation of the internal cycling of nitrogen compounds within a body of water. The study was conducted on lakes of varying trophic states (from oligotrophic to hypereutrophic) in the region around Gainesville, Florida, and thus, has special relevance to lakes in humid, subtropical regions like the southeastern United States where information of this type is especially sparse. However, the results should also have a general significance in other geographical areas, for many aspects of the nitrogen cycle are similar in widely varying climates.

SECTION IV

REVIEW OF THE NITROGEN CYCLE IN NATURAL WATERS

Nitrogen occurs in the biosphere in a variety of forms ranging in oxidation state from +5 to -3. Inorganic nitrogen is present primarily as highly oxidized nitrite and nitrate, as reduced ammonia and as molecular nitrogen. A variety of intermediate gaseous oxides of nitrogen are important in atmospheric chemistry but not in natural waters. Naturally occurring organic nitrogen consists primarily of amino and amide (proteinaceous) nitrogen along with some heterocyclic compounds present as cellular constituents, as non-living particulate matter, as soluble organic compounds, and as inorganic ions in solution. All these forms are interrelated by a series of reactions known collectively as the "nitrogen cycle," which portrays the flow of nitrogen from inorganic forms in soil, air and water into living systems and then back again into inorganic forms.

This cyclic phenomenon can be illustrated diagramatically in various ways: Figure 1 presents a simplified reaction sequence of the interconversions between organic nitrogen and the main inorganic forms, and it labels the principal reactions. This figure indicates the central role of ammonia in the cycle as the link between the organic and inorganic phases. The major reactions of the nitrogen cycle are (1) ammonia and (2) nitrate assimilation, (3) ammonification, (4) nitrification, (5) denitrification, and (6) nitrogen fixation. Reactions (1) and (2) represent and major pathways for conversion of inorganic nitrogen into organic (cellular) forms. Reaction (3) represents a series of complex reactions breaking down organic forms (proteins, amino acids, nucleic acids, etc.) into ammonia. Reaction (4), nitrification, is the aerobic oxidation of ammonia to nitrite and nitrate. Reactions (1) - (4) can be considered to comprise a potentially closed system of reactions (the internal cycling of nitrogen among its various forms within a body of water). Reactions (5) and (6) are respectively *in situ* sinks and sources for nitrogen within given ecosystem. Nitrogen fixation (reaction 6) is the reduction of molecular (atmospheric) nitrogen to ammonia and then to cellular (organic) nitrogen. Molecular nitrogen is not normally considered part of the nitrogen reserve in an ecosystem since it (N_2) is not utilizable by most organisms: thus fixation is a source of nitrogen to the exosystem. Denitrification (reaction 5) is a nitrogen sink since it converts utilizable nitrogen in the forms of nitrate and nitrite to molecular nitrogen under anoxic conditions.

Figure 2 illustrates the nitrogen cycle as it may occur in an idealized stratified lake. With the exception of ammonia exchange with sediments it is apparent that all reactions are biologically mediated. By far the

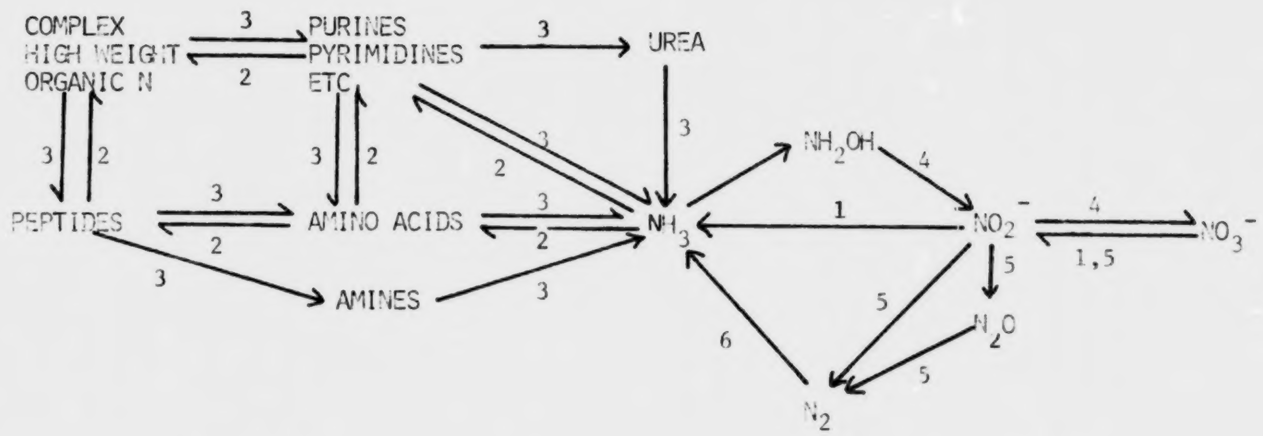
COMPLEX HIGH WEIGHT ORGANIC N
PURINES PYRIMIDINES ETC
UREA
PEPTIDES
AMINO ACIDS
AMINES
NH_3
NH_2OH
NO_2^-
NO_3^-
N_2O
N_2
3
2
1
4
5
6
1,5

FIGURE 1. SIMPLIFIED NITROGEN CYCLE SHOWING MAIN MOLECULAR TRANSFORMATIONS:

1. NITRATE ASSIMILATION, 2. AMMONIA ASSIMILATION, 3. AMMONIFICATION, 4. NITRIFICATION, 5. DENITRIFICATION, 6. NITROGEN FIXATION

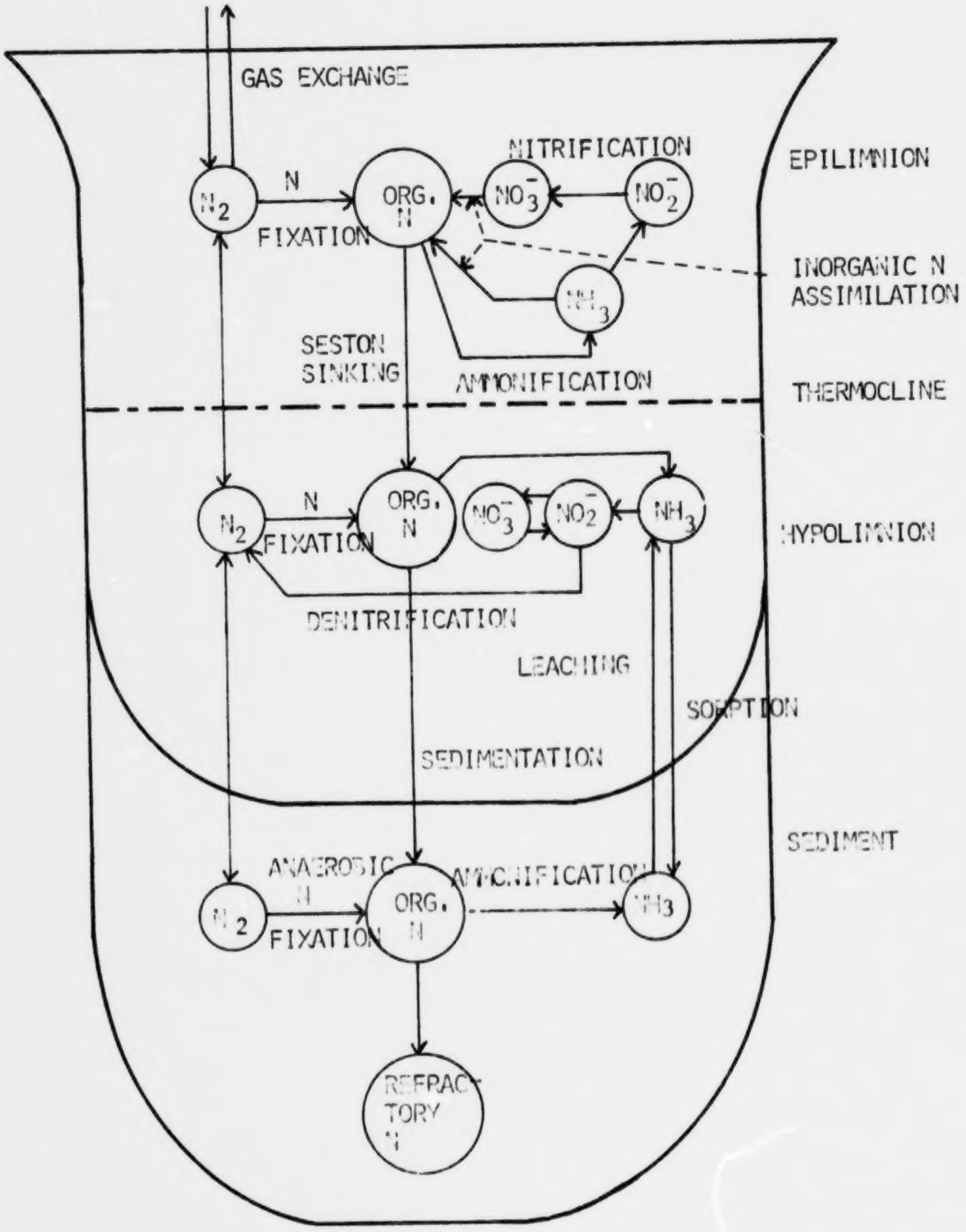
GAS EXCHANGE
NITRIFICATION
EPILIMNION
N_2
N
FIXATION
ORG. N
NO_3^-
NO_2^-
INORGANIC N
ASSIMILATION
NH_3
SESTON
SINKING
AMMONIFICATION
THERMOCLINE
N_2
N
FIXATION
ORG. N
NO_3^-
NO_2^-
NH_3
HYPOLIMNION
DENITRIFICATION
LEACHING
SORPTION
SEDIMENTATION
SEDIMENT
ANAEROBIC
N
FIXATION
N_2
ORG. N
AMMONIFICATION
NH_3
REFRACTORY N

FIGURE 2. NITROGEN CYCLE REACTIONS IN AN IDEALIZED STRATIFIED LAKE.
NOTE THAT BOTH AEROBIC AND ANAEROBIC TRANSFORMATIONS ARE SHOWN IN THE HYPOLIMNION. IN A REAL LAKE THEY WOULD OF COURSE NOT OCCUR SIMULTANEOUSLY. REDRAWN AND ADAPTED FROM KUZNETSOV (1959).

greatest influx of inorganic nitrogen into organisms results from ammonia and nitrate assimilation. These reactions predominate in surface waters and are mediated primarily by phytoplankton and macrophytes. While nitrate tends to be the predominant inorganic nitrogen form in surface waters, there is considerable evidence that ammonia is the preferred form for planktonic assimilation since it is already at the reduction level of organic nitrogen. Nitrate can be used by most plants (Strickland, 1965, and others cite some flagellates as exceptions and a few algae have been reported to prefer nitrate over ammonia (Proctor, 1957)). Organisms using nitrate as their nitrogen source must first reduce it to the level of ammonia before incorporating it into organic forms, which process requires a reduction system including the enzyme nitrate reductase. This inducible enzyme is present in algal cells only when nitrate is being used as the nitrogen source (Eppley, Coatsworth et al., 1969), which suggests a mechanism for determining the form of nitrogen an algal population is using.

Reduction of nitrate also requires the oxidation of organic matter to CO_2, and this oxidation-reduction sequence is apparently not linked to the respiratory chain and to ATP synthesis. Thus utilization of nitrate is in a sense energetically wasteful.

Ammonia is a weak base:

$$NH_4^+ \rightleftarrows NH_{3\,aq.} + H^+ \; , \quad pKa = 9.3.$$

Thus in poorly buffered waters where photosynthesis may raise the pH as high as 10-11, the predominant form is $NH_{3aq.}$, while in well buffered and in low productivity systems the cationic form (NH_4^+) dominates. The fact that ammonia assimilation can continue at acidic pH values (where $[NH_{3aq.}]$ is negligible) implies that the cation itself is transported through the cell membrane. At high concentrations NH_3 is toxic to organisms and this fact may explain the reports of earlier workers that ammonia is a less suitable source of nitrogen for algal cultures than is nitrate. Growth media frequently contain 5 or more mg N/l (as ammonia or nitrate). If CO_2 uptake by photosynthesis is sufficient to raise the pH to 10 or so, then the NH_3 could act as a toxicant. On the other hand assimilation of ammonia and its incorporation into organic matter tends to lower the pH (Fogg, 1965) since NH_3 is a base and its removal tends to increase acidity. Whichever phenomenon is in fact responsible for the inhibitory effects noted with high ammonia concentrations, it is clear that a well buffered system is essential to maintain proper growth conditions in ammonia containing media.

Rates of ammonia and nitrate assimilation in aquatic environments have recently been determined by several workers using ^{15}N tracer techniques. Such studies are relevant to an increased understanding of the metabolism of the aquatic community since the availability and utilization of nitrogen profoundly influences and in some cases controls primary production. Of interest in this regard is such information as the rates of inorganic nitrogen turnover, the relative nutritional importance of the various nitrogen forms, the effect of concentration on rates of utilization, and the relationship between nitrogen assimilation and primary production. Although the ^{15}N tracer approach to studying aquatic nitrogen cycle dynamics is only about 10 years old, considerable gains have already been made through its use. The nitrogen cycle can no longer be viewed as a slow, primarily seasonal cycle. Turnover times for inorganic nitrogen are on the order of days or even hours in many waters, which implies the essentiality of rapid organic nitrogen decomposition or a constant influx of new nitrogen into the system.

Dugdale and Dugdale (1965) were the first to report _in situ_ ammonia and nitrate assimilation rates in surface waters. Using ^{15}N techniques they found two main pulses of assimilation in Sanctuary Lake, Pennsylvania. During the first pulse, in June, ammonia was assimilated most rapidly with a maximum rate of about 110 μg N/l.-day. The maximum nitrate assimilation rate during the same period was about 40 μg N/l-day. A second pulse of assimilation in early September utilized primarily N_2. Some of the high assimilation rates reported may be an artifact of methodology. For example, the nitrate concentration at the time of the June assimilation maximum was only about 10 μg N/l. The amounts of $^{15}NO_3^-$ and $^{15}NH_3$ added to samples were not specified, but a large amount of tracer relative to the initial unlabeled nitrogen would stimulate assimilation beyond the normal rate.

Rates of ammonia and nitrate assimilation in Lake Mendota, Wisconsin, during 1966 were reported by Brezonik (1968, 1971a). Ammonia assimilation rates were greater than nitrate assimilation rates in all cases, and highest assimilation rates in the surface water occurred in late spring and late summer. No correlation was found between ammonia and nitrate concentrations and assimilation rates. Depth profiles of ammonia assimilation during holomixis and early stratification were fairly uniform and no trends were indicated. However, by early June a pronounced stratification of assimilation was found with high rates in the epilimnion and much lower rates in the hypolimnion.

In nearly every assimilation study published thus far ammonia was by far more important than nitrate as a nitrogen source. In view of the ease with which ammonia can be assimilated this is not surprising, but prior to these tracer studies most marine biologists considered nitrate to be the only significant nitrogen source in the sea. It now seems certain that both marine and fresh water algae derive most of their nitrogen from ammonia often in spite of higher nitrate concentrations. There may be a certain amount of "wheel-spinning" involved in ammonia assimilation; rather than representing new production, at least part of the uptake may derive from the necessity to recapture nitrogen from compounds which apparently continually leak through cell walls (Whittaker and Feeney, 1971; Hellebust, 1965; Stewart, 1963). Dugdale and Goering (1967) derived a simple model for the nitrogen cycle in marine surface waters in which they considered primary production associated with ammonia assimilation to be "regenerated" production, and primary production associated with nitrate assimilation was regarded as "new" production. Only the latter would be available for export to higher trophic levels since nitrate input from the deep water is the principal source of nitrogen to the surface. Ammonia in sea water is the product of short term regeneration, and primary production associated with its assimilation is essentially that needed to maintain the standing crop. Extension of this approach to the more complicated nitrogen cycles in shallow lakes would be of perhaps questionable validity.

An exception to the general rule of more rapid ammonia than nitrate assimilation was recently reported by Goering et al. (1970) in the eastern subtropical Pacific. Phytoplankton in a nutrient-rich discontinuity layer at the bottom of the euphotic zone were found to use nitrate primarily while algae in the impoverished surface water used ammonia.

Dugdale (1967) has developed a model for nutrient limitation in the sea in which assimilation of ammonia and nitrate were assumed to follow Michaelis-Menten kinetics:

$$v = \frac{VS}{K_t+S} ,$$

where v is the rate of nutrient assimilation; V a maximum rate, constant under a given set of conditions; S is nutrient concentration; and K_t a transport or half-saturation constant with units of concentration. The numerical value of K_t is equal to the nutrient concentration at which v is one-half the maximum for the system (V/2). Subsequent ^{15}N measurements on natural populations by MacIsaac and Dugdale (1969) and direct uptake measurements on algal cultures by Eppley, Rogers et al. (1969) have verified this model and estimated K_t values for various marine surface waters. K_t values calculated by the former authors ranged from less than 0.2 to 4.2 μg.-atom/l. for nitrate and from 0.1 to 1.3 μg.-atom/l. for ammonia. Lower transport constants correlated with oligotrophic (nutrient-depleted) areas while higher K_t values were found in nutrient rich waters. It was thus suggested that phytoplankton in oligotrophic waters are adapted to low ambient concentrations and can assimilate nutrients more rapidly under these conditions than can phytoplankton from nutrient-enriched regions.

The reverse of assimilation is ammonification, whereby organic nitrogen is returned to the inorganic nitrogen pool as ammonia. This is a complicated process involving several mechanisms. Early workers considered only bacterial decomposition of soluble organic nitrogen and organic detritus as important (vonBrand et. al., 1937), but more recent studies have shown significant excretion of ammonia and amino acids by zooplankton feeding on phytoplankton and detritus. Johannes (1969) suggested this as the dominant mechanism of ammonification in surface waters and reviewed previous work indicating that net zooplankton release amounts of dissolved nitrogen and phosphorus equal to their total body content of these nutrients in 20 to 200 hours. A third mechanism for ammonification is direct autolysis after cell death, which may account for 30 to 50 percent of the nutrients released from plant and animal material (Johannes, 1969; Krause, 1964; Golterman, 1960). The excretion of amino acids and other small nitrogenous substances from photosynthesizing cells (Hellebust, 1965) and their subsequent direct uptake by other algae or bacteria represents a short-circuiting complication of ammonification pathways, the significance of which is not yet understood.

Current information and theories suggest that bacterial decomposition of organic matter accounts for a minor portion of nutrient regeneration in marine surface waters and in shallow lakes (although this is probably not not true in systems receiving large organic inputs from sewage outfalls). In soils and bottom sediments and in anoxic hypolimnia of lakes, nutrient regeneration by bacteria and fungi is the dominant process. Ammonification is obviously important in reviewing a limited supply of nitrogen for assimilation and growth of primary producers.

In situ ammonification rates can be measured by an isotope dilution technique (Zilversmit et al., 1943; Dugdale, 1965; Brezonik, 1968). Assuming steady state conditions for assimilation and ammonification, ^{15}N label added to the ammonia pool will gradually become diluted as both labeled and unlabeled ammonia is assimilated but comparatively unlabeled N is returned to the ammonia pool by ammonification of organic N. The decrease in ^{15}N enrichment in the ammonia pool can be quantified and related to the rate of ammonification. Because of the difficulties encountered in ^{15}N measurements, only a few in situ ammonification rates in lakes have been reported. Alexander (1970) reported that maximum ammonification rates in Smith Lake, Alaska, were associated with a spring bloom of Anabaena and with the period of maximum ammonia assimilation. Relatively high rates were measured in Lake Mendota, Wisconsin (Brezonik, 1968, 1971a) with a tendency for higher rates in the bottom waters. From steady-state kinetics, ammonia turnover times were calculated to range from 7 to 62 hours in Lake Mendota surface waters during the summer. Because steady state conditions do not exactly apply and because of the errors inherent in the methodology, these calculations are only approximate (Brezonik, 1971a); nonetheless they suggest a much more rapid cycling of nitrogen than measurement of concentration changes alone would suggest.

Ammonia is oxidized to nitrite and nitrate in the process of nitrification by a select group of aerobic autotrophic bacteria which obtain their energy by nitrogen oxidation and their cellular carbon by reduction of CO_2. A variety of heterotrophic bacteria, actinomycetes and fungi (Schmidt, 1954; Hatcher and Schmidt, 1971) have also been reported capable of nitrification, generally at much slower rates, but the significance of these organisms in aquatic nitrification is not well known. The biochemistry and metabolism of nitrifying organisms has been reviewed by Painter (1970) particularly with reference to its occurrence in sewage treatment. The significance of nitrification in the nitrogen cycle lies in the conversion of labile ammonia (which tends to be lost from solution by sorption onto sediments and by volatilization at high pH) to a more stable form (nitrate). On the other hand, nitrate can be reduced to molecular nitrogen by the process of denitrification, and thus nitrification has a second and opposite role -- that of producing the reactants for this nitrogen sink. The oxidation of ammonia to nitrate requires almost 4.5 mg O_2 per mg of ammonia - N oxidized. Thus nitrification can act as an important oxygen sink in streams which receive high ammonia concentrations from unnitrified sewage effluents. Attempts to quantify and model these effects have recently been described by a number of workers (Stratton and McCarty, 1967; Wezernak and Gannon, 1969; Thomann et al., 1971).

In situ nitrification rates can be estimated by adding $^{15}NH_3$ to a water sample and determining the amount of label in the nitrate and nitrite fractions after incubation (Brezonik, 1968). The latter is accomplished by reducing the oxidized nitrogen to ammonia with Devarda's alloy (Bremner and Keeney, 1966), and subsequent conversion of ammonia to N_2 by hypobromite oxidation (Rittenberg et al., 1939; Neess et al., 1962) for isotope ratio analysis. Dugdale and Goering (1967) were unable to detect nitrification in the surface waters of the Sargasso Sea using incubations as long as a week, and it appears that regeneration of nitrate in the sea occurs primarily below the euphotic zone. A few nitrification rates were reported for Lake Mendota by Brezonik (1968, 1971a). During 1966 rates increased with depth in spring but were very low in the surface water during summer. The fact that nitrate levels invariably decline in lake surface waters during spring and summer suggests that nitrifying bacteria are unable to compete with algae for ammonia, and nitrification is thus felt to be of minor significance in surface waters during the growing season (Brezonik, 1968). However, Brezonik and Lee (1968) also found that substantial nitrification evidently occurred at mid-depths (7-17 m.) in Lake Mendota, Wisconsin, during late spring and early summer. Largest increases occurred in the 12-15 meter zone where nitrate increased from less than 0.2 mg. N/l. in June to 0.5-0.7 mg. N/l. in late July. Nitrification in the hypolimnion is especially significant since the water eventually becomes anoxic during late summer, and denitrification takes place. Thus nitrification increases the importance of denitrification as a nitrogen sink in Lake Mendota. Vollenweider (1963) has also reported nitrification at mid-depths during summer stratification in Lake Orta, Italy.

In the process of denitrification nitrate is used as a terminal electron acceptor by facultative and anaerobic bacteria in the absence of oxygen. Nitrite is formed as the first intermediate in the process, and nitrous oxide can sometimes be formed along with molecular nitrogen although it is not an essential intermediate. Since the principal end product (N_2) is a nitrogen form not utilizable by most organisms, this reaction acts as a nitrogen sink and can assume importance in the nitrogen balances of lakes and other aquatic systems subject to intermittent anoxia, (e.g. soils, Delwiche, 1965). The biochemistry of denitrification has been reviewed extensively (Delwiche, 1956, 1965; Kessler, 1965; Painter, 1970), who describe the long and considerable controversy over the requirement of completely anoxic conditions for this reaction to occur. The consensus now is that denitrification occurs only when oxygen is absent from the system or at least sufficiently low enough to allow anoxic microzones to develop.

Denitrification may account in part for the difficulty encountered in obtaining nitrogen balances in waste treatment plants (Wuhrmann, 1954; Symons et al., 1965). The reaction may occur in anoxic microzones of activated sludge floc or in anoxic settling basins. Nitrification followed by intentional denitrification has been proposed as a means of nitrogen removal from biological waste treatment plants (Johnson and Schroepfer, 1964; Wuhrmann, 1964). Denitrification has been shown to be an important nitrogen sink in lakes (Brezonik and Lee, 1968; Goering and V. Dugdale, 1966). Its role in the nitrogen cycle of the oceans is less defined but there is no doubt of its occurrence in some oceanic environments (Goering and R. Dugdale, 1966; Goering, 1968).

Not all the nitrate that is reduced is lost to the system as N_2. A significant but variable amount of assimilatory reduction to ammonia and organic nitrogen occurs simultaneously (but different organisms may be involved in the two processes). This was first noted by this author in denitrification experiments with sewage sludge digestors; only 50 percent of added nitrate was recovered as N_2 (Brezonik and Lee, 1966). An early study of denitrification in lakes (Goering and V. Dugdale, 1966) suggested that assimilatory nitrate reduction was unimportant based on low measured rates of ammonia production from labeled nitrate into organic nitrogen. Brezonik and Lee (1968) found that reduction to ammonia and organic nitrogen occurred at comparable but somewhat lower rates than denitrification in Lake Mendota, Wisconsin. Of the 4.43×10^7g., nitrate-N present in the lake hypolimnion below 14 m. in mid summer of 1966, an estimated 2.81×10^7g. were lost by denitrification while the balance (1.62×10^7g.) was reduced to ammonia and organic nitrogen. Goering (1968) also reported ratios of N_2-N formed/NO_3^--N lost ranging from 1/10 to 8/10 in the eastern Pacific, indicating that assimilatory reduction is of great but variable importance.

Denitrification in lake sediments may be an important nitrate sink in ground water seeping into lakes as Keeney _et_ _al_. (1971) have recently described for Lake Mendota, Wisconsin. Finally, denitrification may be be the mechanism whereby the high N/P ratios of fresh waters are transformed to the lower ratios encountered in estuarine waters (Stumm, 1971). No _in situ_ experiments of denitrification have been conducted in such environments, but if density stratification is sufficient to produce anoxic or near anoxic conditions in the bottom layers of an estuary, this reaction could occur.

Probably more attention has been devoted to nitrogen fixation than to all the other nitrogen cycle reactions combined. This process is important at several levels. Geochemically nitrogen fixation is essential in maintaining a nitrogen balance in the biosphere, which would otherwise become depleted as a consequence of denitrification over a time scale of millions of years. In agriculture the reaction is important in maintaining or increasing soil fertility. In natural waters nitrogen fixation acts as a source of nitrogen and permits continued organic production when the supply of fixed nitrogen becomes depleted. A variety of organisms are capable of nitrogen fixation including a number of blue-green algae, apparently all photosynthetic bacteria, various aerobic bacteria (e.g. Azotobacter), anaerobic bacteria (e.g. _Clostridium_), many facultative bacteria (but only under anoxic conditions (Wilson, 1969)), legume root nodules and nonleguminous root nodulated plants such as _Podocarpus_ and the alder tree (_Alnus_ sp.). Most studies of nitrogen fixation in natural waters to date have emphasized the role of filamentous, heterocystous blue-green algae. The occurrence of _Azotobacter_ and _Clostridium_ in the Black Sea (Pshenin, 1959, 1963) and in other aquatic habitats has been established, and photosynthetic bacteria have been found in dense populations in a variety of lakes (e.g. Trüper and Genovese, 1968). However the presence of bacteria capable of fixation does not necessarily imply the actual occurrence of fixation in any given environment. On the other hand these organisms have the potential for fixation presumably because it is useful to them at least in some habitats and it would be surprising if fixation by these organisms were not found in the environment. Most biologists have de-emphasized the role of these organisms (especially the heterotrophic bacteria) in nitrogen fixation because they have felt that insufficient oxidizable carbohydrate (or other substrate) would be available in the environmental Stewart (1966, 1968, 1970) has reviewed the agents and occurrence of nitrogen fixation in the biosphere in greater detail.

Nitrogen fixation is generally considered to be an adaptive process used by organisms only when the supply of fixed nitrogen is depleted. Nitrogen fixing algae usually bloom in lakes only after nutrients have been depleted by blooms of other algae (i.e. late summer in temperate lakes). However, contrary to earlier opinions, small to moderate concentrations of ammonia do not necessarily inhibit fixation although synthesis of the enzyme nitrogenase is repressed at high levels. Stewart (1969) suggests that the levels of combined nitrogen nitrogen in most natural ecosystems are insufficient to inhibit fixation immediately or even to persist long enough for existing nitrogenase to be diluted out. Low levels of combined nitrogen may actually be advantageous to nitrogen-fixing plants by enabling more efficient and healthy growth than could be achieved on N_2 alone.

Reduction of N_2 to the level of ammonia requires energy and a source of reduced hydrogen, both of which may be obtained either from photosynthetic production or from oxidation of organic carbon. Nitrate reduction to ammonia similarly requires energy obtainable by oxidizing organic substrates. While the net reactions in each case are exergonic, they are nevertheless energy sinks since the cells apparently have no means of trapping and storing the energy (as ATP) given off by the reactions. To this extent use of nitrate and of molecular nitrogen rather than ammonia for assimilation is wasteful of energy.

Molybdenum has been shown to be a specific requirement for organisms using nitrate or fixing nitrogen, and it apparently is a constituent of the enzymes directly involved in the processes (nitrate reductase and nitrogenase, respectively). This element has been shown to be a limiting nutrient in various soils and at least one lake has demonstrated a deficiency. Goldman (1960) found a planktonic growth response when molybdenum was added to Castle Lake, California; this Mo limitation was presumably linked to nitrogen metabolism. Nitrogen fixing organisms also require iron in greater amounts than is required by organisms growing on fixed nitrogen, and cobalt (or vitamin B_{12}) has also been implicated as an essential nutrient for fixation.

The biochemical pathway of nitrogen fixation is poorly understood. A long history of efforts to isolate intermediate reduction products is recorded in the literature, but thus far only ammonia -- the final inorganic intermediate before conversion to organic nitrogen compounds -- has been definitely shown to be a step in the process. Because of this distinct lack of success in finding intermediates, most biochemists currently favor a pathway in which the intermediates are tightly bound to the enzyme active site. Burris (1966, 1969) has reviewed the biochemistry of fixation in several informative articles.

Suggestions that nitrogen fixation occurs in lakes date back at least as far as far as Hutchinson (1941), who thought that Anabaena was responsible for an increase in fixed nitrogen in Linsley Pond (Connecticut) during the summer. The first in situ measurements of lacustrine fixation were reported by Dugdale et al. (1959). These workers developed the ^{15}N technique (Neess et al. 1962) and demonstrated the occurrence of low rates of fixation in several lakes. More thorough studies were reported by these workers for several Alaskan lakes (Dugdale and Dugdale, 1961) where only very low rates were detected; in Sanctuary Lake, Pensylvania, (Dugdale and Dugdale, 1962) where a maximum rate of 130 μg N fixed per liter per day was associated with a late summer bloom of Anabaena; in Lakes Mendota and Wingra, Wisconsin, (Goering and Neess, 1964); in Smith Lake, Alaska (Dugdale, 1965); and in the Sargasso Sea (Dugdale et al., 1964). High rates of fixation have been consistently correlated with blooms of blue-green algae, primarily Anabaena, Gloeotrichia, and Aphanizomenon in lakes, Trichodesmium in subtropical marine waters and Calothrix in shallow temperate marine environments (Stewart, 1965).

The expense and difficulties associated with ^{15}N tracer techniques precluded routine and detailed investigations of nitrogen fixation in aquatic environments until recently when a simple, indirect method of assessing nitrogen fixation rates was developed (Stewart et al., 1967). This method utilizes the fact that the nitrogen fixing enzyme complex reduces acetylene to ethylene, the production of which can be sensitively and easily determined by gas chromatography. Acetylene ($H-C\equiv C-H$) is isoelectronic with molecular nitrogen ($N\equiv N$), and the nitrogenase system is incapable of distinguishing between the two molecules. In fact a number of other similarly shaped molecules such as cyanide $(C\equiv N)^-$ and carbon monoxide ($C=O$) also react at or attach to the nitrogenase active site and thus act as enzymatic inhibitors of fixation (Burris, 1969). Based on the fact that nitrogenase reduces acetylene to ethylene ($H_2C=CH_2$), Dilworth (1966) proposed that di-imide ($HN=NH$) is the first intermediate in the reduction of nitrogen. Of the various compounds that react at the nitrogenase active site, acetylene is best suited for indirect assay of nitrogenase activity. Neither acetylene nor its reduction product is toxic to the organisms. Furthermore acetylene reduction yields a single, easily measured product; (ethylene is not further reduced to ethane by the enzyme). Finally the affinity of nitrogenase for acetylene is large, in fact larger than for its normal substrate. Thus even in the presence of molecular nitrogen, acetylene is preferentially reduced. The advantages of the acetylene reduction assay over ^{15}N techniques are both a greatly simplified methodology and a greater sensitivity. These properties have facilitated such fundamental studies on aquatic nitrogen fixation as the effects of sunlight intensity, detailed depth profiles, and diel variations (Stewart et al., 1967; Rusness and Burris, 1970). In addition the fact that acetylene reduction yields a gaseous product has enabled the study of low fixation rates in lacustrine and estuarine sediments (Brooks *et al.*, 1971; Keirn and Brezonik, 1971); see Section VI.

In work supported by this project Brezonik and Harper (1969) reported evidence for nitrogen fixation in anoxic lake hypolimnia, and Stewart (1969) has similarly reported on fixation in anoxic waters of a Norwegian fjord. Brooks (1969), Brooks *et al.*, (1971) and Keirn and Brezonik (1971) in work described later in this report have also found low but measurable rates of nitrogen fixation in estuarine and lacustrine sediments, and Howard *et al*. (1970) have reported low fixation rates in Lake Erie sediments. Thus the known distribution of nitrogen fixation in aquatic habitats has been extended considerably by recent work.

The rate at which newly fixed nitrogen is transferred from agents of fixation to other aquatic organisms is of great interest. Jones and Stewart (1969) have reported that extracellular nitrogen liberated by the marine nitrogen fixer, *Calothrix scopulorum*, is assimilated by marine algae, fungi, and bacteria and could serve as the sole nitrogen source for a species of *Chlorella*. Based on limiting nutrient bioassays, Fitzgerald (1969) concluded that transfer of nitrogen newly fixed by *Aphanizomenon* is of little significance to colonies of *Microcystis* growing in the same water, but this is perhaps an overextension of the data. It seems clear from Fitzgerald's results that *Aphanizomenon* is unable to supply *Microcystis* with sufficient nitrogen to satisfy the organisms, but the actual magnitude of supply is still unclear.

With the exception of sediment-water interactions (to be discussed in a later section), the major nitrogen cycle reactions of natural waters have been discussed above. As presently understood the nitrogen cycle is almost exclusively a biological phenomenon. However, the possible importance of chemical reactions and of heretofore undetected (but potential) biological transformations should not be overlooked. A variety of other reactions are thermodynamically feasible, and some of these could have a substantial impact on the overall cycle. The mechanisms of N_2 formation from fixed nitrogen have been the subject of a long controversy and many pathways have been proposed and rejected. Formation of N_2 directly from ammonia has been hypothesized to occur in sludge digestion (Malina, 1961; Crane, 1962) and anoxic lake waters (Koyama, 1964), but others have found no evidence for such a reaction (Brezonik and Lee, 1966; Wijler and Delwiche, 1954). The latter authors used ^{15}N methods to show that all N_2 in denitrification derives from oxidized nitrogen (NO_3^-,NO_2^-) sources. The reaction of nitrous acid with amino groups under acid conditions:

$$RCHCOOH + HNO_2 \longrightarrow RCHOHCOOH + N_2 + H_2O$$

(commonly known as the Van Slyke reaction) has been proposed by some workers to be significant in acid lakes (Hutchinson, 1957) and soils (Reuss and Smith, 1965) but has been rejected by others (Bremner and Nelson, 1968; Nelson and Bremner, 1969). However the latter authors have provided evidence for significant chemical decomposition of nitrite to a variety of gaseous products in soils. Bremner and Nelson (1969) demonstrated that nitrite decomposition in sterilized soils is due to reaction of nitrite with soil organic matter and to self decomposition of nitrous acid ($2HNO_2 = NO + NO_2 + H_2O$) under acid conditions. The nitrite-organic matter reaction was found to involve phenols and polyphenols such as lignins and tannins. The mechanism is thought to involve reaction of nitrous acid under slightly acid conditions (pH 5) with phenolic compounds to form nitrosophenols which tautomerize to quinone oximes. The latter are decomposed by nitrous acid to form N_2 and N_2O (Austin, 1961). This reaction could be of importance in the nitrogen cycle of natural waters. Colored lakes are high in polyphenolic substances (tannins, lignins, "humic acid") and generally have acid pH values. While nitrite levels in natural waters are normally low, such a reaction could act to decompose nitrite as rapidly as it if formed.

While photochemical reactions are highly important in the atmospheric chemistry of nitrogen, photochemistry is presently thought to play an insignificant role in aquatic nitrogen transformations. Since most photochemical reactions are induced by UV light, such processes should be limited to a narrow surface layer. Photochemical nitrification in the sea was proposed by various workers in the 1930's (ZoBell, 1935; Rakestraw and Hollaender, 1936; Cooper, 1937) partly as a result of difficulties in culturing marine nitrifiers (Waksman et al., 1933), but Hamilton (1964) concluded that such a reaction is of no significance in the marine nitrogen cycle. Hamilton found slight photoreduction of nitrite but concluded that this too was insignificant.

Several thermodynamically possible nitrogen reactions have never been shown to occur as biological phenomenon. For example, N_2 could be used as a terminal electron acceptor in organic carbon oxidation with the release of sufficient energy for organism growth:

$$\text{glucose} + 4N_2 + 8H^+ + 6H_2O = 6CO_2 + 8NH_4^+ \text{ , } \Delta G^o_w = -84.5 \text{ kcal/mole.}$$

The above reaction of course occurs when heterotrophic organisms fix nitrogen, but it is not thought that any of this energy is trapped and retained by nitrogen fixing cells for other uses. However, it is conceivable that this reaction could be a source of energy for some anaerobic bacteria. Oxidation of molecular nitrogen to nitrate by molecular oxygen is also exergonic at pH 7:

$$N_2 + \frac{5}{2}O_2 + H_2O = 2NO_3^- + 2H^+ \text{ (w), } \Delta G^o_w = -15.2 \text{ kcal/mole.}$$

With the large reservoir of available reactants for this process, it is surprising that no organisms have evolved to take advantage of the situation. However, this is probably fortunate considering the nature of the product (i.e. nitric acid).

SECTION V

TRANSPORT OF NITROGEN INTO LAKES

The concentration and forms of nitrogen in a lake at a given time are a product of input rates, of the interconversion reactions occurring within the lake and the rates of loss via outflow, denitrification and sediment deposition. Lakes act in general as nutrient traps, implying an accumulation of nitrogen in bottom sediments as a lake gradually fills in. Both natural and cultural nitrogen sources can be significant in lacustrine nitrogen budgets. The natural nutrient input to a lake is a function of its drainage basin geochemistry (i.e. the potential for nutrient leaching from soils and substratum), drainage basin size, hydrology, precipitation patterns, etc. (Brezonik et al., 1969). Superimposed on these factors are a multitude of human or cultural factors, which can be expressed in terms of drainage basin land use patterns and population characteristics. Quantitative information on lacustrine nitrogen budgets and the significance of individual sources is sparse, but concern over cultural eutrophication has stimulated much needed measurements of the nitrogen (and phosphorus) contributions from various sources along with development of elementary nutrient models.

Table 1 lists the possible nitrogen sources and sinks which must be considered in calculating the nitrogen budget for a lake. The classical approach to evaluation of a lake's nutrient budget is actual measurement of nutrient concentrations and flows for each source over a reasonable time span (e.g. one year). Obviously this requires a large time and manpower expenditure, especially for large lakes, and some diffuse sources (e.g. ground water seepage) may not be amenable to direct or accurate evaluation. Because of these difficulties it is probable that complete measurements of all sources and sinks have not been accomplished for any single lake although approximate nutrient budgets have been established for a few American and European lakes (see Brezonik et al., 1969, and Vollenweider, 1968, for reviews). An alternative to actual measurement is development of a simple simulation model of nutrient transport based on knowledge of the lake's drainage basin size, population, hydrology and land use patterns and on literature values for nutrient contributions from the various sources. This approach was taken by Lee et al. (1966) in their well-known budget for Lake Mendota, Wisconsin. Actual measurements of nutrient contributions are undoubtedly more accurate than literature estimates, but as the literature on nutrient export rates from various land uses and other sources becomes more complete, the accuracy of literature-based nutrient budgets will increase. The accuracy of such nutrient budgets depends not only on the accuracy of the original measurements but also on their applicability to the particular system under consideration. However considering the expense and difficulties of direct

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Table 1. Sources and Sinks for the Nitrogen Budget of a Lake

Sources

1. Surface

   - Agricultural (cropland) runoff and drainage
   - Animal waste runoff
   - Marsh drainage
   - Runoff from uncultivated and forest land
   - Urban storm water runoff
   - Domestic waste effluents
   - Industrial waste effluent
   - Wastes from boating activities

2. *In situ*

   - Nitrogen fixation
   - Sediment leaching

3. Airborne

   - Rainwater
   - Aerosols and dust
   - Leaves and miscellaneous debris

4. Underground

   - Natural ground
   - Subsurface agricultural and urban drainage
   - Subsurface drainage from septic tanks near lake shore

Sinks

- Effluent loss
- Groundwater recharge
- Fish harvest
- Weed harvest
- Insect emergence
- Volatilization (of NH_3)
- Evaporation (aerosol) formation from surface foam)
- Denitrification
- Sediment deposition of detritus
- Sorption of ammonia onto sediments

measurement, literature-based models seem the only reasonable approach for many lakes receiving cultural stress. Thus refinement of nutrient contribution values from the various types of sources is of great importance.

Clearly experimental evaluation of the quantitative importance of the myriad sources of nitrogen was beyond the scope of the present project. Rather it was concerned primarily with the importance of in situ sources and sinks, especially nitrogen fixation and sediments (see Sections VI-VIII). However, in conjunction with a concurrent study of eutrophication in Florida lakes a significant effort has been made to gather information from the literature regarding the significance of natural and cultural sources and to apply this information to the development of nitrogen budgets and simple nutrient-eutrophication models for Florida lakes. In addition the significance of rainfall as a nutrient source received some experimental attention during this project.

The significance of particular nitrogen sources has received considerable attention in various recent reviews, and an exhaustive literature review is not in order herein. For details the reader should consult the original literature or the specific reviews cited below. This section will present a summary of the nitrogen (and to a lesser extent phosphorus) contributions from various sources and describe the use of such data in developing nutrient budgets and enrichment models. Feth (1966) reviewed the sources and concentration range of nitrogen in natural waters emphasizing natural sources and a geochemical viewpoint. Agricultural sources, especially fertilizer and soil losses have been treated by Biggar and Corey (1969), and C. F. Cooper (1969) has reviewed nutrient export from forest land. Weibel (1969) reviewed his and other studies on urban nutrient sources. Schraufnagel et al. (1967) and Lee et al. (1966) reviewed the natural and cultural sources of nitrogen especially as applied to lakes and streams in Wisconsin, and Vollenweider (1968) has provided a broad ranging review on both nitrogen and phosphorus sources for lakes.

Table 2 summarizes the ranges of nitrogen and phosphorus contributions from various natural and cultural sources and provides further references which have evaluated or reviewed their significance. Nutrient contributions from undisturbed forest land are generally considered to be small, but fertilization and clear-cutting tend to increase nutrient export (Cole and Gessel, 1965; Bormann et al. 1968). For example, the former authors found that nitrogen output via percolotion water from a Douglas fir forest increased from 0.54 kg./ha. to 0.69 and 1.04 kg./ha. when plots were fertilized with urea and ammonium sulfate, respectively. Clear-cutting increased nitrogen export to 0.96 kg./ha., and this practice also tends to increase runoff and sediment transport. Bormann et al.(1968) have found large alterations in the nitrogen cycle of a watershed after its hardwood forest was leveled and regrowth prevented by herbicide applications. Greatly increased nitrate levels were noted in stream water (greater than 10mg. N/l.), and nitrogen export rose from less than 2 kg./ha.-yr. to more than 6.0. Thus properly managed and undistrubed forests retain their accumulated nutrients efficiently and not surprisingly should be of little concern in eutrophication control. However management and harvest practices can alter this state of affairs and produce considerable nutrient export.

Table 2. Nitrogen and Phosphorus Contents
of Various Nutrient Sources[1]

Source	N	P	References
Natural			
Forest runoff	1.3-5.0 1.5-3.4	.084-.18 .83-.86	Cooper, 1969; Sylvester, 1961
Forest Percolation water	0.54	.034	Cole & Gessel, 1965
Swamp and marsh runoff	?	?	Vollenweider,1968 Lee *et al.* 1971
Meadowland and runoff	?	?	Vollenweider,1968
Precipitation on lake surface	.58 .18-.98	.044 .015-.060	Brezonik *et al.*, 1969 Vollenweider, 1968 Hutchinson, 1957, Feth 1966; Weibel *et al.*,1966b
Aquatic birds	.48-.95	.09-.18	Sanderson, 1954 Paloumpis and Starrett, 1960; Gates, 1963
Leaves and pollen	?	?	Vollenweider, 1968 Goldman, 1961, Hynes Kaushik, 1969, Richardson, *et al.*, 1970
Cultural			
Domestic sewage	39.4	0.80	Vollenweider, 1968 Sawyer, 1947; Mackenthun *et al.* 1964;Englebrecht&Morgan, 1959
Agricultural areas Citrus	22.4	0.18	Montelaro,1970
Pasture	8.5 1-5	0.18 0.15-0.75	Miller, 1955 Vollenweider,1968
Cropland	5-120	0.22-1.0	Johnson,et.al,1965 Moe 1967; Biggar&Corey, 1969
Farm animals, feedlots	?	?	Vollenweider, 1968; B. A. Stewart, 1970; Loehr, 1969; Miner *et al* 1966; Hutchinson & Viets, 1969
Urban runoff	8.8	1.1	Weibel *et al*, 1964;1966ab Weibel, 1969; Palmer, 1950; Sawyer, *et al*, 1945
Septic tanks	?	?	Polta, 1969; Patterson *et al*, 1971
Marsh and landfill drainage	?	?	Quasim, 1965

[1] All units in kg/ha-yr. except rainfall (g/m^2-lake area-yr.), birds (kg/duck-yr.), and domestic sewage (kg/capita-yr.).

Undisturbed meadowland is probably camparable to forestland in retention of nutrients, but intensively used pastureland appears to be a somewhat greater source. In many areas pasture land is fertilized to increase the yield of grass, and this fertilizer coupled with animal wastes undoubtedly increases nutrient export rates. Swamp and marshland have frequently been cited as important both in trapping nutrients before they reach a lake and in contributing to the nutrient budget of a lake (Benson, 1965; Lee 1970a), but quantitative information is sparse. Recently Lee et. al. (1971) described the annual nutrient balance in several Wisconsin marshes as in approximate steady state with the nitrogen and phosphorus income equal to the outflow. During growing season in these temperate marshes nutrient inflow is retained by the marsh but these nutrients appear to be exported during a short period of high flow in spring. On the other hand, draining a marsh and converting it to dry land tends to release large amounts of nitrogen and phosphorus as a result of soil oxidation and mineralization. Pollen was recently shown to be an insignificant source of nitrogen for Lake Tahoe (Richerson et. al., 1970), but it may be of greater importance (a few percent or so) in small alpine or subalpine lakes with limited nutrient inflow and greater shoreline to volume ratio. Leaves are known to exert a significant effect on water quality in streams (Slack and Felz, 1968), but quantitative assessment of their role in lacustrine nutrient budgets is a difficult matter.

Nutrient contributions of water fowl are not well known but may be significant in some cases, especially where large rookeries and migratory bird preserves occur on small water bodies. A community of about 50 mallard ducks maintained for research purposes on the shore of Lake Mize, a small, deep lake near Gainesville, Florida, was recently estimated to have increased the nitrogen and phosphorus loading rates beyond those suggested as critical for eutrophication (Brezonik, 1971b). Duck contributions were calculated to represent 50 and 75 percent of the nitrogen and phosphorus loading rates of 8.1 and 0.73 g./m^2-yr., respectively. Waste production of small and medium sized birds (ducks, chickens) have been assessed in relation to pollution from commercial breeding, production, and processing (Sanderson, 1954; Gates, 1963). The role of wild aquatic birds in the nutrient regime of a water body may be more that of cycling agents than of direct sources (i.e. much of their food may be taken from the lake itself - fish- or from the immediate watershed). However, such activities can still stimulate increased production and apparent eutrophication by accelerating nutrient cycling and degrading a normally more stable (organic) nutrient reservoir.

Agricultural sources of nitrogen are difficult to summarize because of large local variations in soil retention capacities, in irrigation and fertilization practices, and in differences associated with climate and type of crops grown. However, some data are available to indicate the significance of agricultural runoff as a nutrient source. Johnston et. al. (1965) found large percentages of nitrogen applied to crop lands in the San Joaquin Valley of California appeared in the tile drainage effluents. From 9 to 70 percent and phosphate concentrations ranged from 0.053 to

0.23 ppm., but even these comparatively low concentrations are sufficient to stimulate algal growths. Moe et al. (1967) found considerably smaller losses of nitrogen from soils in southern Indiana. A maximum of 15 per cent of applied fertilizer (NH_4NO_3) was found in surface runoff after simulated heavy rains.

Waste output for the common farm animals (cattle, hogs, sheep, horses) are reasonably well known (Vollenweider, 1968; B. A. Stewart, 1970), and total animal populations are readily available in areal units at least as small as counties. However, the amount of animal waste which enters and is transported by the source waters to a lake depends largely on local circumstances, and few quantitative figures are available. Ammonia volatilized from cattle feed lots has been shown to contribute significantly to the nitrogen budgets of down wind lakes (Hutchinson and Viets, 1969). For example, this source was estimated to add about 0.6 mg. NH_3-N/1.-yr. to Seely Lake, Colorado, a small lake 2 km. down wind from a 90,000 unit cattle feed lot. Ground water in areas of cattle feed lots is frequently contaminated with high nitrate levels, but present data are insufficient to allow prediction of nitrogen fluxes for individual situations.

Nitrogen contributions from sewered populations are well known on a per capita basis, and the total nitrogen concentration in raw and treated domestic sewage is probably sufficiently constant to permit evaluation of this source for nutrient budget purposes from readily available sewage flow and population records. Non-sewered human waste contributions, as from septic tank drainage, are far more difficult to evaluate. Septic tanks are undoubtedly important factors in the cultural eutrophication of many recreational lakes. However there are almost no data available to quantify this source (see Patterson et al. 1971 for a detailed discussion). Urban runoff composition has been studied in detail in a few places (Weibel et al., 1964, 1966a; Palmer, 1950; Sawyer et al. 1945), and storm drainage flows are more widely known, but the general applicability of existing information outside the original study areas is unknown.

Rainfall has recently received attention as a source of nutrients, especially nitrogen. Menzel and Spaeth (1962) correlated the ammonia content of Sargasso Sea surface water with the rainfall of the previous five days, and Gambell (1963) found rainfall to be a major source of nitrate and sulfate in certain Virginia and North Carolina streams. Chalupa (1960) found significant rainfall contributions of phosphate to a Czech reservoir. Parker (1968) has found bloom stimulating concentrations of vitamin B_{12} in rainfall of the St. Louis area. Weibel et al. (1964, 1966a,b) reported extensive studies on rainfall and urban and rural runoff as nutrient sources in the Cincinnati, Ohio, region. Cincinnati rainfall averaged more than one mg/l in total nitrogen and 0.24 mgP/l in hydrolyzable phosphate. Reviews on rainfall as a nutrient source have been compiled by Feth (1966), Hutchinson (1957) and Vollenweider (1968), and if previous studies have confirmed one point, it is that nitrogen concentrations in rainfall are (unfortunately) highly variable. Both nitrate and ammonia occur in significant amounts but the latter is usually higher. In the tropics nitrate is said to be somewhat more important than in temperate rains but even there ammonia is usually higher (Hutchinson, 1957). Total nitrogen concentrations frequently approach 1/mg. N/l., and both natural and cultural sources are responsible. The correlation of high rainfall ammonia levels with alkaline soils and of low rainfall ammonia content in regions with acid soils suggests that sorption of ammonia onto soil clays may be an important factor in the hydrospheric nitrogen cycle (Feth, 1966). Although a number of cultural sources (ammoniated fertilizers, nitrogen oxides in auto exhaust) would seem to make important contributions to atmospheric fixed nitrogen, there is conflicting evidence regarding the correlation of high rainfall nitrogen levels with areas of industrial or cultural activity (see Vollenweider, 1968, and Feth, 1966, for details). Careful analysis of historical records to discern possible long term trends toward increased rainfall nitrogen resulting from intensified inorganic fertilizer use, from increases in vehicular traffic and internal combustion engines (sources of nitrogen oxides), or from other cultural sources has not been reported.

While relatively large amounts of data have been collected on nitrogen in rainfall in several areas of the country, little information is available for the Florida peninsula. Because of the variabilities in concentrations and amounts, it is not yet possible to make reliable estimates of rainfall contributions to nutrient budgets without a thorough field study. To further knowledge concerning these variabilities several studies on nutrients in rain were conducted during the project. The great variations in amount of rainfall within a small geographical area are indicated in Table 3. Eight stations scattered 8 miles long were set up in August, 1968, and 9 stations were similarly established during the rainy season in August, 1969. Rainfall was collected for 7 days in 1968 and during 3 days of intermittent showers in 1969, in pyrex bottles through 4 inch diameter glass funnels (see Appendix A for analytical details). The results from 1968 show an inverse correlation between rainfall amounts and nutrient content, in agreement with the role of rainfall as a cleansing agent for the atmosphere. The marked variations in rainfall amounts even in this small geographic area were surprising. Whether the sampling stations would have such large differences over longer periods is not known, but presumably the differences would become small over the period of a year or so. Ammonia and nitrate were much higher in the 1968 collections than in 1969, which may be related to differences in amounts of rainfall during the days preceding sampling in the two years. Since rainfall seems to cleanse the atmosphere a large amount of rain prior to a collection period would result in lower concentrations in collected samples. The ratio of nitrate to ammonia varied markedly among samples in both years; this would seem to reflect the importance of local terrestrial sources such as dust and airborne fertilizer since the larger scale atmospheric movements and patterns of circulation would tend to homogenize rainfall composition over as small an area as sampled here. It is interesting to note that orthophosphate concentrations in the samples were with few exceptions higher than the critical concentration said to stimulate blooms in lakes. Thus, rainfall might stimulate blooms rather than providing a dilution effect; this seems especially likely during summer when surface waters are nutrient depleted.

Table 3. Variations in Nutrient Content of Rainfall in Gainesville Area[1]

Sampling Station	Volume Collected[2]	Ortho PO_4-P	t-PO_4-P	NH_3-N	NO_2^--N	NO_3^--N
			August, 1968			
1	700	0.03	--	0.08	0.004	0.25
2	370	0.23	--	0.79	0.024	0.41
3	150	0.40	--	0.28	0.006	0.40
4	120	0.13	--	0.81	0.007	0.52
5	115	0.07	--	0.27	0.007	0.52
6[3]	35	0.06	--	0.79	0.018	0.94
7[3]	27	0.29	--	1.26	0.008	1.11
			August, 1969			
A	250	0.006	.033	0.35	--	0.02
B[3]	135	0.043	0.65	0.10	--	0.09
C	290	0.009	0.125	0.20	--	0.11
D	20	0.018	--	0.05	--	0.07
E	130	0.011	0.076	0.14	--	0.07
F	180	0.018	0.061	0.02	--	0.07
G	130	0.004	0.045	0.05	--	0.05
H[3]	290	0.021	0.32	0.10	--	0.05
I	170	0.002	0.022	0.15	--	0.06

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[1] Concentrations in mg/l.

[2] Volume in ml collected in 1 liter pyrex bottle with 4 inch diameter glass funnel over 7 day period in 1968 and 3 day period in 1969.

[3] Sample contained large amounts of particulate matter evidently from dry fallout

While Gainesville is not heavily industrialized, the high nutrient levels in Table 3 may be partly the result of man's activities. To determine the nutrient concentrations in rural rainfall, a series of sampling stations were set up near Cedar Key, Florida, a small town on the Gulf of Mexico about 65 miles from Gainesville. Eight stations were set up within the town and on a line from the Gulf up to 20 miles from the coast. Results for a rainfall in late August, 1968, are shown in Table 4. Ammonia and nitrate were considerably lower in these rain samples indicating possible cultural effects in the Gainesville samples. However, the Gainesville rains were brief intense showers, while the Cedar Kay rain was steady fine rain lasting for 24 hours. Also the former samples were collected over a week's time while the latter samples represent 24 hours of rain. The effect of saline Gulf water on the ionic content (especially Na$^+$) of rainfall is clearly seen in the first two stations on Cedar Key. These were the only stations with detectable ammonia, but nitrate and phosphate show no trends with distance from the coast.

Previous studies (Putnam and Olson, 1960; Weibel et al., 1964) have described rainfall as a cleansing agent for the atmosphere. Nutrient and particulate concentrations in precipitation were generally found to decrease during the course of an extended rainfall and to increase in rain with the length of antecedent drought. Several experiments were conducted in this project to study the washing out of airborne nutrients over short periods of time during rainfalls. Rainwater was collected on the roof of the Environmental Engineering Building at various time intervals (five minutes to one hour) after the beginning of showers on several occasions during August and September, 1969. Samples were collected in pyrex bottles with a large funnel and preserved with mercuric chloride for analysis of nitrogen and phosphorus forms.

Results from three rain showers (Table 5) indicate a rapid cleansing of nutrients from the atmosphere. A fourth rainfall (Table 5) did not show such marked decreases; this was a light steady rain rather than an intense shower, and it was preceded by a heavy rain on the previous day. Most of the phosphorus in rain was present as ortho-phosphate. Initial concentrations were considerably higher than the threshold levels for algal bloom problems, but after ten minutes or so, the concentrations dropped to 0-10µg P/l. Organic nitrogen and ammonia were the predominant forms of nitrogen in the rain, and initial levels were comparable to those in eutrophic waters. Both forms generally decreased to 0.1mg N/l or less within an hour. Nitrate levels were lower -- no more than 0.08 mg N/l initially -- and declined to trace levels rapidly. A trace of nitrite was found on only one occasion. The rapid decline of nutrient forms in rain showers suggest that the nutrients may originate as low altitude aerosols. If this is the case, the high initial concentrations probably reflect local, cultural activity (e.g. auto exhaust, airborne fertilizer from plowed fields, etc.), whereas the low concentrations afterward may reflect natural or general levels of nutrients in the atmosphere.

It should be apparent from the above results that estimation of total nutrient contributions to a lake via rainfall is not so simple a matter as one might have first thought. In particular, the use of literature values for nitrogen concentrations is likely to produce inaccuracies

Table 4. Nutrient Content of Coastal and Rural Rainfall[1]

Location[2]	Amount[3]	NH_3-N	NO_3^--N	O-PO_4-P	Ca^{+2}	Mg^{+2}	Na^+	K^+
Cedar Key a	580	0.08	0.05	0.012	1.34	3.05	11.0	1.7
Cedar Key b	530	0.03	0.05	0.036	0.61	0.24	2.0	0.3
1	490	0.00	0.04	0.010	--	--	--	--
3	490	0.00	0.06	0.007	--	--	--	--
5	640	0.00	0.03	0.027	0.30	0.05	0.26	0.08
10	770	0.00	0.05	0.028	0.31	0.04	0.38	0.15
15	1070	0.00	0.05	0.024	0.22	0.05	0.45	0.18
20	710	0.00	0.06	0.010	0.31	0.03	0.31	0.12

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[1] Concentrations in mg/l.

[2] Stations were located at the waters' edge on Cedar Key (a), on the island interior (b), and on a line approximately due east from Cedar Key with the distances (in miles) from the coast as noted.

[3] Amount in ml collected in 1 liter pyrex bottles with 4" diameter glass funnels during 24 hour period.

Table 5. Temporal Variations of Nutrients in Rain Showers in Gainesville, August and September, 1969[1]

Date	Time[2]	o-PO_4-P	t-PO_4-P	TON	NH_3-N	NO_3^--N
4 Aug.	Type of rain: thunder shower following weekend of dry weather					
	0-5	0.056	0.058	1.85	0.92	0.08
	5-10	0.032	0.032	1.00	0.84	0.08
	10-20	0.010	0.015	0.83	0.09	0.00
	20-30	0.005	0.005	0.10	0.09	0.00
	30-60	0.005	0.005	0.15	0.10	0.00
10 Sept.	Type of rain: shower following day with trace of rain					
	0-5	0.080	0.150	0.85	0.85	0.08
	5-10	0.031	0.031	0.75	0.75	0.05
	10-20	0.010	0.015	0.10	0.10	0.01
	20-30	0.00	0.00	0.15	0.10	0.01
	30-60	0.00	0.00	0.09	0.10	0.01
16 Sept.	Type of rain: thunder shower following day with light shower					
	0-5	0.038	0.045	1.60	0.50	0.05
	5-10	0.010	0.010	1.30	0.40	0.01
	10-20	0.010	0.010	0.0	0.18	0.00
	20-30	0.00	0.00	0.0	0.0	0.00
	30-60	0.00	0.00	0.0	0.13	0.00
3 Sept.	Type of rain: light and steady following day with 2 inches of rain					
	0-5	0.008	0.008	0.15	0.10	0.08
	5-10	0.005	0.005	0.10	0.10	0.005
	10-20	0.00	0.00	0.00	0.08	0.00
	20-30	0.00	0.00	0.00	0.00	0.00
	30-60	0.00	0.00	0.10	0.00	0.00

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[1] All concentrations in mg/l

[2] Time in minutes after start of rainfall

because of the large local and temporal variations which characterize the nitrogen content of rain. For phosphorus in rainfall there are almost no data available in the literature, so this flux must be evaluated experimentally. In conjunction with a concurrent project on eutrophication factors in Florida lakes, the total nitrogen and phosphorus contributions of rainfall to the nutrient budget of a small lake were determined in 1968. A recording rain gauge was established at the lake site 30 miles east of Gainesville, and samples were collected at weekly intervals for ammonia, nitrite, nitrate and phosphate analyses. Rain was collected in a pyrex bottle with a 4-inch diameter glass funnel. In order to prevent contamination of collected rainfall by dust, leaves, dead insects and other particulate matter a wad of glass wool was placed over the funnel drain. Results of this study are summarized in Table 6. Because of the inverse correlation between nitrogen content and amount of rainfall, use of a simple mean value of total N for all measurements times the volume of rainfall on the lake surface would not be very accurate. Consequently nitrogen contributions were calculated for each sampling increment and these results then summed. Rainfall is a significant although not dominant nutrient supplier to Anderson-Cue Lake. If the artificial nutrient input to this lake is not considered, rainfall then is seen as an even more important source, especially for oligotrophic lakes in nutrient impoverished watersheds (as is Anderson-Cue Lake).

In summary, a large volume of raw data on nutrient sources is available, but generalizations on the importance of various sources can in only few cases be estimated. A few point sources (e.g. domestic sewage) are reasonably well characterized, and some diffuse sources (e.g. urban runoff) can probably be estimated with fair accuracy using literature values. However the quantitative aspects of nutrient budgeting are still relatively undeveloped.

From a management point of view it is more important to determine the contributions from man-made sources since they are more readily controlled than natural sources. However the importance of natural sources must be known in order to determine whether control of man-made sources will result in significant improvements in water quality. The most significant cultural sources of nitrogen are domestic sewage, agricultural (cropland) runoff, animal wastes from farming operations, and urban runoff. Nitrogen fixation, rainfall, ground water, and natural stream flow are probably the most significant natural sources. Undoubtedly sediment deposition, flow through the outlet (in lakes) and denitrification are the largest sinks for nitrogen in natural waters. Outlet losses would seem to be directly proportional to the nitrogen concentration within the body of water. Denitrification is limited in lakes to those that stratify thermally and lose their oxygen in the bottom water.

The collected information on nutrient export from various sources has been used in conjunction with land use and population data to compute nitrogen and phosphorus budgets for 55 lakes in north and central Florida (Shannon, 1970; Shannon and Brezonik, 1971a). Because this work was performed primarily in relation to a concurrent project (Brezonik, 1971c), detailed results are not presented here. However, the findings are of interest in relation to the preceding discussion of nitrogen sources, and a summary

Table 6. Contribution of Rainfall to Nutrient Budget of Anderson-Cue Lake

Total rainfall in 1968:	136.7 cm (53.8 in)
Lake area:	7.7×10^4 m^2
Lake volume:	1.85×10^5 m^3

Rainfall nutrients in g/m^3

Nutrient	Mean	Range
TON	0.31	0.07-0.67
NH_3-N	0.21	0.01-0.86
NO_3^--N	0.21	0.04-0.94
Ortho PO_4-P	0.009	0.002-0.033
Total PO_4-P	0.033	0.020-0.070

Budget

	N		P	
Source	kg	g/m^3	kg	g/m^3
Rainfall[1]	49.7	0.27	3.4	0.019
Artificial nutrient mixture[2]	124	0.67	10.6	0.057
Other sources (surface runoff)[3]	134	0.72	4.4	0.024
Total	308	1.66	18.4	0.100
Percent of total contributed by rain	16		18	

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[1] Rainfall nitrogen levels were inversely proportional to amount of rain collected; budget value was calculated from total N measured at each collection times the amount of rain collected since last analysis and summing these amounts over the entire lake surface. Since relatively few total phosphate analyses were run and no inverse correlation with rainfall amount could be discerned, rainfall phosphorus in the budget was calculated from the mean for all samples (0.033 g/m^3) times the total 1968 rainfall volume over the lake surface.

[2] Ammonium chloride and sodium phosphate dissolved in sewage as a controlled enrichment experiment (Brezonik et al., 1969).

[3] Calculated from watershed (land use) patterns from Shannon (1970) and Shannon and Brezonik (1971a).

is presented below. Watershed land use patterns were obtained from recent aerial photographs, lake and watershed areas were measured from U.S.G.S. topographic maps, and population estimates for each watershed were obtained by counting the dwellings visible on aerial photographs. Nitrogen budgets were then calculated from appropriate export rates (i.e. kg N exported per hectare of a particular land use per year, kg N per capita per year etc.) and the measured watershed parameters. Budgets were calculated per unit area of lake surface and also per volume of lake water. On an areal basis nitrogen loading rates ranged from 1.2 g./m.^{2}-yr., for a small oligotrophic lake (Swan Lake, Putnam County) to over 90 g./m.^{2}-yr., for Lake Alice, a shallow, hypereutrophic lake which receives 1.5-2.0 mgd of treated sewage from the University of Florida waste treatment plant. Expressed on a volumetric loading basis, nitrogen extremes were 0.18 to 106 g./m.^{3}-yr.

Loading rates generally correlated with lake trophic conditions. In order to study relationships between lake conditions and watershed characteristics (e.g. nutrient loading rates), a numerical index (TSI) was developed from values for 7 quantitative trophic indicators (e.g. primary production, total N and N levels, Secchi disc transparency). Annual mean values for the parameters from the 55 Florida lakes and the multi-variate statistical rechnique of principal component analysis were used in this derivation (Shannon and Brezonik, 1971b). Increasing values of the index corresponded to increasingly eutrophic conditions, and various analyses of the results suggest the index does in fact quantify the concept of trophic state.

Regression analyses of the trophic state index (TSI) vs. N and P loading rates (expressed on both areal and volumetric bases) were performed using an additive model (TSI = f(N + P) and a multiplicative model (TSI = f(N + P). Some representative results are given in Table 7. In that phosphorus was incorporated into most of the stepwise regression equations first and was hence the most important variable in a statistical sense, it might be inferred that phosphorus rather than nitrogen is the controlling input for eutrophication in Florida lakes. However, regression analyses are inherently empirical, and such inferences must be approached with [illegible]

Of great interest in control of cultural eutrophication is the development of critical loading rates for nitrogen and phosphorus, above which one can expect eutrophic conditions to ensue. Sawyer (1947) was the first to propose quantitative guidelines of this sort. Based on data from Wisconsin lakes he suggested that 0.015 mg/l of inorganic phosphorus and 0.3 mg/l of inorganic nitrogen at the spring maximum are critical levels, above which algal blooms can normally be expected. In the absence of any other studies, these values have been widely quoted and applied to many types of lakes in diverse geologic and climatic situations. Recently Vollenweider (1968) analyzed the available data on nutrient loading rates and corresponding trophic conditions and proposed permissible and critical loading rates for nitrogen and phosphorus as a function lake mean depth. Using the regression equations shown in Table 7, similar critical loading rates were estimated for Florida lakes (see Shannon and Brezonik, 1971a, or Brezonik, 1971c for details of this derivation). Results are shown in Table 8, and Vollenweider's

Table 7. Regression Analyses of Trophic State Index (TSI) vs Nitrogen and Phosphorus Loading Rates for 55 Florida Lakes (from Brezonik, 1971a)

Model	Loading Rate Units	Equation[a]	F Ratio[b]	Multiple Correlation Coefficient	Percent Variance Explained by Equation
Additive					
(1)	per unit lake surface area	$TSI = 0.62(N_{SL}) + 10.06(P_{SL})$	46.44	0.804	64.5
(2)	per unit lake volume	$TSI = 26.1(P_{VL}) + 0.90(N_{VL})$	43.20	0.793	62.9
Multiplicative					
(3)	Per unit lake surface area	$TSI = 0.84(P_{SL})^{.48}(N_{SL})^{.20}$	14.08	0.600	36.0
(4)	per unit lake volume	$TSI = 1.08(P_{VL})^{.42}(N_{VL})^{.04}$	15.64	0.620	38.5

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[a] Abbreviations: TSI = trophic state index (dimensionless); N_{SL} and P_{SL} = nitrogen and phosphorus surface loading rates in g/m^2-yr; N_{VL} and P_{VL} = nitrogen and phosphorus volumetric loading rates in g/m^3-yr.

[b] All significant at the 99% confidence level.

Table 8. Critical Loading Rates for Nitrogen and Phosphorus

Reference	Loading Rate Units	Permissible Loading (up to)		Dangerous Loading (in excess of)	
		N	P	N	P
Shannon and Brezonik 1971a	Volumetric (g/m^3-yr)	.86	.12	1.51	.22
Ibid.	Areal (g/m^2-yr)	2.0	.28	3.4	.49
Vollenweider (1968)[a]	Areal (g/m^2-yr)	1.0	.07	2.0	.13

[a] For lakes with mean depths of 5 m or less.

(1968) values for shallow lakes ($\bar{z} < 5$m.) are given for comparison. While differences are evident between the two analyses, the estimates are of the same magnitude. Florida lakes appear capable of assimilating somewhat greater quantities of nutrients before becoming mesotrophic or eutrophic than is indicated by Vollenweider's values.

The above values should not be regarded as final. In a sense they are quite unrefined because of uncertainties and even complete ignorance regarding nutrient export rates from various types of terrain and land uses. However, as these values are experimentally determined the empirical analysis described here should become more precise, and it offers the potential for quickly and economically predicting water quality conditions from watershed characteristics.

SECTION VI

NITROGEN FIXATION AS AN IN SITU NITROGEN SOURCE FOR NATURAL WATERS

I. ALGAL FIXATION IN LAKES

Aside from the external nitrogen sources discussed previously, several mechanisms within a lake can effect an increase or decrease in the total N content of the lake. Nitrogen fixation is an in situ source, while denitrification is a sink. Sediments must act as nitrogen sinks over the geological life span of a lake, but over shorter periods of time they may act as sources to the overlying water. The role of sediments as a source or sink and of denitrification is discussed in Section VIII.

A wide variety of studies were undertaken to assess the significance of nitrogen fixation as a nitrogen source in lacustrine systems. For the purpose of this report the studies can be grouped into four phases: 1.) an extensive survey of nitrogen fixation in the 55 Florida lakes described in the previous section, 2.) an in depth study of fixation in two eutrophic lakes, Bivin's Arm and Newnan's Lake, 3) investigations on bacterial fixation in lakes, and 4.) investigation of the nitrogen fixing properties of sediments. The first two phases are described in this section, the latter two in Section VII. Analytical procedures used throughout these studies are described in Appendix A.

In order to estimate the overall significance of nitrogen fixation as a nutrient source, fixation measurements were included as part of a broad ranging study of the limnology and trophic conditions in Florida lakes (Brezonik, 1971c). The 55 lakes included in the study were of widely varying trophic and chemical conditions (Shannon and Brezonik, 1971c), and most are located within a 40 mile radius of Gainesville. All 55 lakes were sampled 4 times, and 19 of them were sampled 7 times over one year. For ease in sample handling, all incubations were carried out under laboratory conditions (22°C in the light) in a shaker-bath; samples were assayed on the same day as they were collected. This procedure undoubtedly yielded results somewhat different from in situ data, but laboratory incubation permitted many more samples to be processed. For assay and comparative purposes, the method is well suited.

Assuming the lakes represent a cross-section of Florida lakes as a whole, it is pertinent to consider the frequency of nitrogen fixation in them. Out of a total of 272 lake samplings (not including separate samples taken in depth profiles on some of the lakes), 42 samples from 15 different lakes gave positive fixation rates using the acetylene reduction method (Table 9). Lake Mize, in which fixation was found only in the anoxic hypolimnetic waters is not included here, but the 7 samplings

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Table 9. Summary of the Occurrence of Nitrogen Fixation in Florida Lakes

Number of lakes sampled:	55
Number of lakes in which fixation was detected:	15
Total lake samplings (not including separate depth profiles on some lakes):	272
Number of positive rates detected:	42
Range of measured rates (nM C_2H_4/l-hr):	1-262
(ng N/l-hr):	93-2450

Frequency distribution of fixation:	#positive samples	#lakes
	1	5
	2	5
	3	0
	4	2
	5	0
	6	2
	7	1

Nitrogen fixing algae detected: *Anabaena* *sp*., *Aphanizomenon* of Newnan's Lake and Bivin's Arm conducted as part of the 55 lake study are included. The infrequent sampling routine probably missed fixation in 4 or 5 more lakes which seem likely environments for sporadic nitrogen fixation (based on their trophic conditions and the nature of their plankton). Low (undetectable) fixation may be more widespread or more frequent in lakes giving occasional high rates, but no attempt was made to concentrate the algae by filtration or centrifugation before incubation. It is doubtful whether such low rates (as requiring organism concentration for detection) would be ecologically significant. Fixation was detected on only one occasion in 5 of the 15 lakes and in 5 other lakes it was found twice. Overall fixation is thus not a typical feature of the lakes. However, in five lakes it apparently occurs commonly; rates in these lakes are summarized in Table 10. Rates expressed as equivalent N fixed in ng. NH_3-N/l.-hr. ranged from just detectable (about 0.5) to a high of 175, but most rates were low (less than 10). However even rather low rates can contribute to a lake's nitrogen budget if the rates continue for a long period. For example, fixation was found in Lake Dora on all 7 sampling dates and in Lake Hawthorne on 6 out of 7 sampling dates. Mean fixation rates in the two lakes are 116 and 127 ng. N/l.-hr. respectively. Assuming this mean rate occurs over a year and that fixation occurs on the average for 8 hours a day, fixation would then contribute about 0.34 and 0.37 mg. N/l. to Lakes Dora and Hawthorne, respectively, on an annual basis.

Detectable fixation was found in the lake surface waters only in the presence of blue-green algae, and the phenomenon seems limited to eutrophic or mesotrophic lakes. None of the oligotrophic Trail Ridge lakes gave detectable fixation, and species of algae capable of fixation were not found in their plankton. Anabaena and Aphanizomenon were the only two nitrogen fixing algae detected in the lakes, and the former was by far the more common.

The seasonal pattern of nitrogen fixation in temperate lakes has been well established (Dugdale and Dugdale, 1962; Goering and Neess, 1964), and fixation is primarily a late summer phenomenon. In subarctic lakes significant fixation is apparently limited to parts of the short ice-free season (Billaud, 1968). In tropical and subtropical lakes seasonal cycles are less pronounced and fixation is possible year-round, but no data are available to substantiate this point. Consequently, a detailed year study of nitrogen fixation was conducted on two highly eutrophic lakes near Gainesville in order to (1) determine seasonal variations in nitrogen fixation, (2) estimate the total contribution of fixation to the nitrogen budgets of these lakes, (3) determine the environmental factors controlling fixation, and (4) relate fixation activities to other biological cycles and phenomena in the lakes, viz. nutrient cycles, primary production, algal blooms.

The two lakes chosen for the detailed study are Newnan's Lake and Bivin's Arm, both of which are located near Gainesville, Florida, and are within 10 miles of each other. Both lakes are highly eutrophic, but the chemical characteristics and causes of these conditions in each lake are quite different. Newnan's Lake is the catchment for a large pine forest and

Table 10. Nitrogen Fixation Rates in Selected Florida Lakes[1]

Date	Bivin's Arm	Lake Dora	Lake Hawthorne	Newnan's Lake	Unnamed #20
1969					
June	67.2	19.6	134	0	0
August	1020	280	392	0	0
October	0	26.6	9.8	19.6	95.2
December	0	133	49	384	47.6
1970					
February	165	57.4	151	133	0
April	16.8	88.2	506	30.8	39.2
June	440	210	0	0	26.6

[1] All rates in ng N/l-hr, derived from nmoles ethylene produced/l-hr. using theoretical factor of 1.5 moles ethylene produced per mole ammonia fixed.

and partially swampy area. The drainage basin north of the lake is rich in phosphatic minerals (Clark et al., 1964), and while some cultural enrichment may enter the lake as runoff from fertilized pasture and leachate from City of Gainesville sanitary landfill operations northeast of the lake, Newnan's Lake appears to be largely naturally eutrophic. The bathymetry of Newnan's Lake is shown in Figure 3; its large surface area (2433 ha.) and shallow depth ($\bar{z}$ = 2m) coupled with its loose, unconsolidated organic sediments insure frequent wind generated resuspension of the surficial sediments and their recirculation to the surface waters. On the other hand, Bivin's Arm receives much of its nutrient budget from cultural sources, including a stream which drains a large urbanized area and into which poorly treated sewage has in the past been dumped, septic tank drainage, and cattle wastes from University of Florida experimental farms on its western edge. A large rookery on the northern shore of the lake may supply substantial nutrients and effect a more rapid cycling of nutrients in the lake. This lake is also shallow ($\bar{z}$ = 1.5m; see Figure 4 for a bathymetric map), and even in the absence of cultural inputs the lake would probably be highly productive. In their presence the lake can only be described as grossly enriched. Algal blooms are present continuously through the year. Secchi disc transparencies greater than 0.6m (2 ft.) are uncommon, and the water frequently has a rather sickly green color.

Table 11 summarizes the chemical characteristics of these two lakes. Newnan's Lake is highly colored from the swamp and pine forest drainage, and its water is both low in conductance and in hardness. The pH of the lake is neutral to slightly alkaline, but the low alkalinity implies a poor buffer capacity. A rather high iron content (average .23 mg/l) is associated with the high color, but manganese is very low as are other heavy metals. Total phosphate in Newnan's Lake, while high and reflecting eutrophy, is less than one-fourth the level in Bivin's Arm. Bivin's Arm has considerably more dissolved solids and hardness which probably arise from cultural sources. Its pH is usually greater than that of Newnan's Lake and its alkalinity is much greater. Organic color is comparatively low and that which is present is at least partially autochthonous.

The biota of these two lakes is somewhat different although blue-green algae are dominant in both. Aphanizomenon is the predominant bloom former during winter in Newnan's Lake whereas Anabaena may bloom in summer; Microcystis is commonly present in abundance in both lakes. Anabaena is the common nitrogen fixing bloom former in Bivin's Arm, but the flora of this lake are much more diverse than in Newnan's Lake, and several pulses of diatom blooms also occur during the year (Harper, 1971). Water hyacinths (Eichornia crassipes) have been a serious problem in both lakes, and their surfaces have been nearly covered by this species within the recent past. Chemical spraying now keeps the growth of hyacinths in check, but the effect of the herbicides employed on other parts of the ecosystems is a matter of question and concern.

Figure 5 shows the concentrations of major nitrogen forms, rates of nitrogen fixation and primary production in Bivin's Arm from May, 1969,

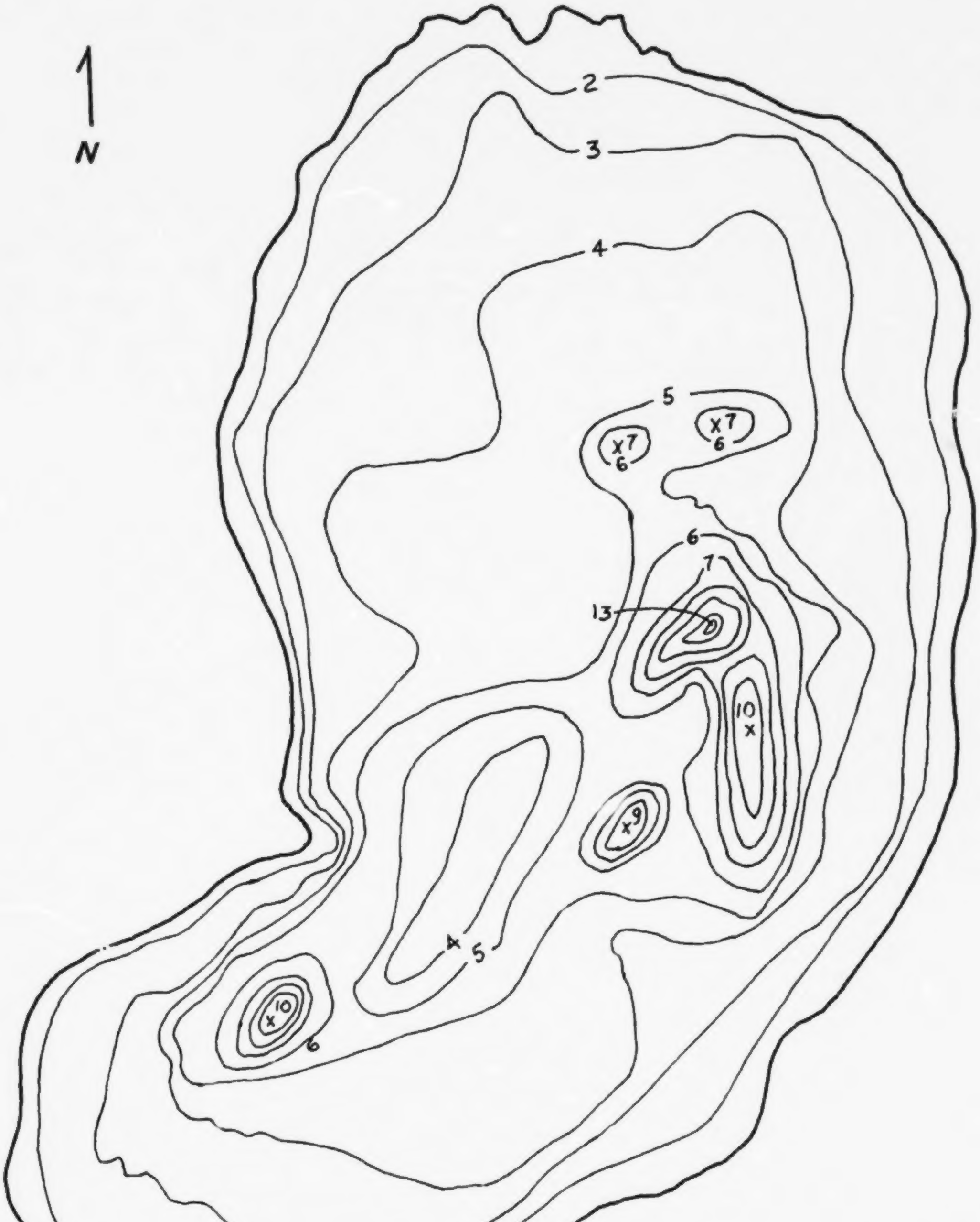
N
2
3
4
5
X7
6
X7
6
6
7
13
10
X
X9
4
5
X10
6
0
1000 M.

FIGURE 3. BATHYMETRIC MAP OF NEWNAN'S LAKE, ALACHUA COUNTY, FLORIDA. DEPTH CONTOURS IN FEET.

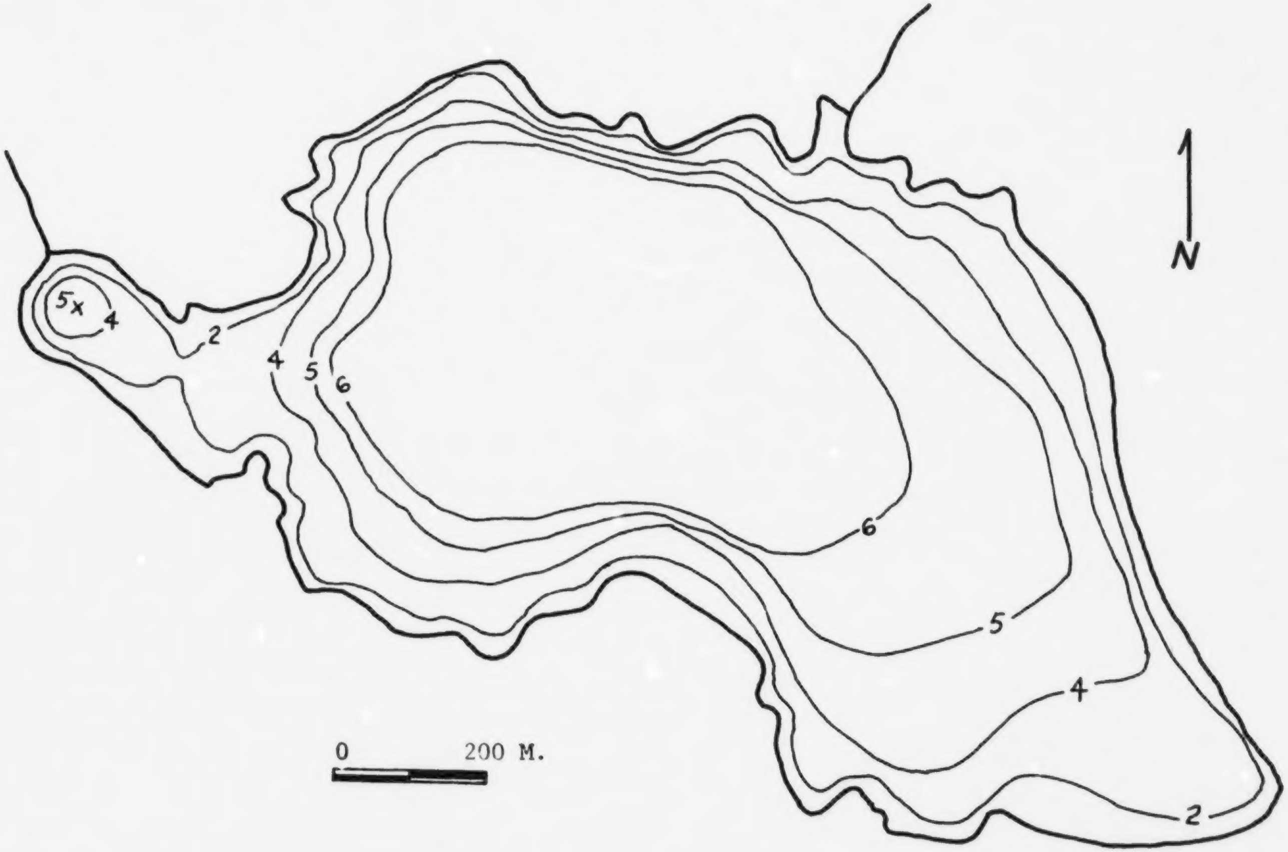
N
5x
4
2
4
5
6
6
5
4
2
0
200 M.

FIGURE 4. BATHYMETRIC MAP OF BIVIN'S ARM, ALACHUA COUNTY, FLORIDA. DEPTH CONTOURS IN FEET.

Table 11. Chemical Characteristics of Newnan's Lake and Bivin's Arm[1]

Parameter[2]	Newnan's Lake	Bivin's Arm
Dissolved oxygen	8.6	10.0
pH	7.8	9.0
Alkalinity (as $CaCO_3$)	7.3	105
Acidity (as $CaCO_3$)	1.0	0
Conductivity (μ mho cm^{-1})	65.7	267
Turbidity (JTU)	4.8	11
Color (as Pt)	235	48
Total organic N	1.55	1.94
Total P	0.10	0.45
Cl^-	11	16
$SO_4^=$	3	7
Na^+	8.4	11.5
K^+	0.6	1.7
Mg^{+2}	1.5	4.7
Ca^{+2}	5.3	30.3
Fe	0.23	0.04
Mn	0.002	0.002
COD	68	60

[1] Mean values for six samplings (every 2 months) over the period June, 1969 to April, 1970.

[2] Concentrations in mg/l except as noted.

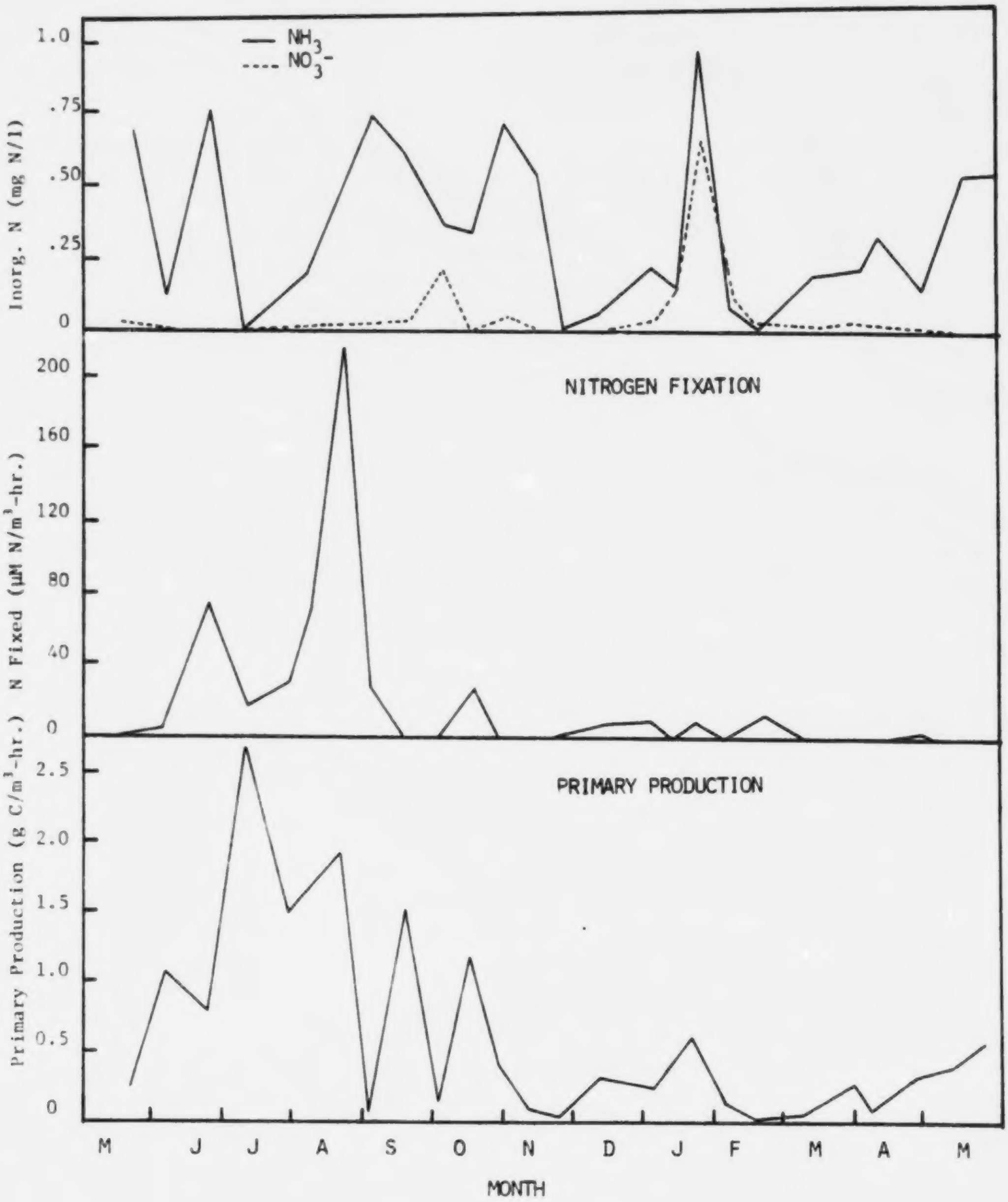

FIGURE 5. INORGANIC NITROGEN LEVELS, NITROGEN FIXATION AND PRIMARY PRODUCTION IN BIVIN'S ARM, FLORIDA, FROM MAY, 1969 TO MAY, 1970.

to May, 1970. Routine data for each sampling data are listed in Appendix B. Fixation occurred at moderate to high rates throughout the summer of 1969; a maximum of 330 nmoles ethylene/l.-hr. (equivalent to about 3.1μg. N/l.-hr.) was found in late August. Lower rates were noted during winter, and fixation was detected on about 50 percent of the sampling dates. Fixation rates in this lake do not seem to be significantly correlated with either primary production or nitrogen concentrations. A rather definite seasonal trend in primary production is apparent, with peak rates in summer (June to October) and lower rates from November to May, but the pattern of algal activity is really much more complex, being characterized by a pronounced short-term variation in both production and nutrient levels.

Seasonal patterns of nitrogen forms, fixation rates and primary production in Newnan's Lake are displayed in Figure 6, and routine data collected on each sampling date are listed in Appendix B. In spite of the geographical proximity and similar enriched conditions in the two lakes, their patterns of fixation are quite dissimilar. Except for one high rate of nitrogen fixation in July, 1969, during a bloom of Anabaena and Microcystis and for very low rates on several occasions in spring and fall, fixation in Newnan's Lake is associated with a dense winter and early spring bloom of Aphanizomenon. In winter of 1969-1970 the bloom occurred in two pulses. The first pulse began in mid-December and died out in early January, apparently because of adverse weather (an abrupt cold wave and an extended period of heavy rain). The population stayed low in January and gradually increased during February to high levels through most of March. Maximum fixation rates during the Aphanizomenon bloom were about 40 nmoles ethylene/l.-hr. roughly equivalent to 0.37μg. N/l.-hr. Fixation was highly correlated with primary production during the winter bloom period (Figure 7) in Newnan's Lake, but no obvious correlation exists between fixation and concentrations of nitrogen forms.

It should be noted that algal fixation occurred in both lakes in the presence of moderate to high (i.e. for lake waters) ammonia levels. In fact the maximum rate measured in Bivin's Arm occurred when the ammonia concentration was 0.5 mgN/l. High rates in Newnan's Lake occurred at ammonia levels as high as 0.6 mgN/l. Thus while fixation occurs frequently on nutrient depleted waters, it is not limited to them. Previous studies have demonstrated simultaneous assimilation of ammonia, nitrate and molecular nitrogen (Dugdale and Dugdale, 1965; Billaud, 1968); no doubt this also occurs in the lakes studied here.

Several measurements of areal variability in nitrogen fixation and related parameters were made on Newnan's Lake during the project in order to evaluate the homogeneity (or lack thereof) of biogenic parameters in the lake and hence determine the number of sampling stations required for adequate representation of the lake. Table 12 presents the results of an areal study conducted on April 19, 1969. Top, mid and bottom samples were composited at 10 stations scattered around the entire lake. All samples were collected within one hour and brought to shore for processing. Primary production and nitrogen fixation samples

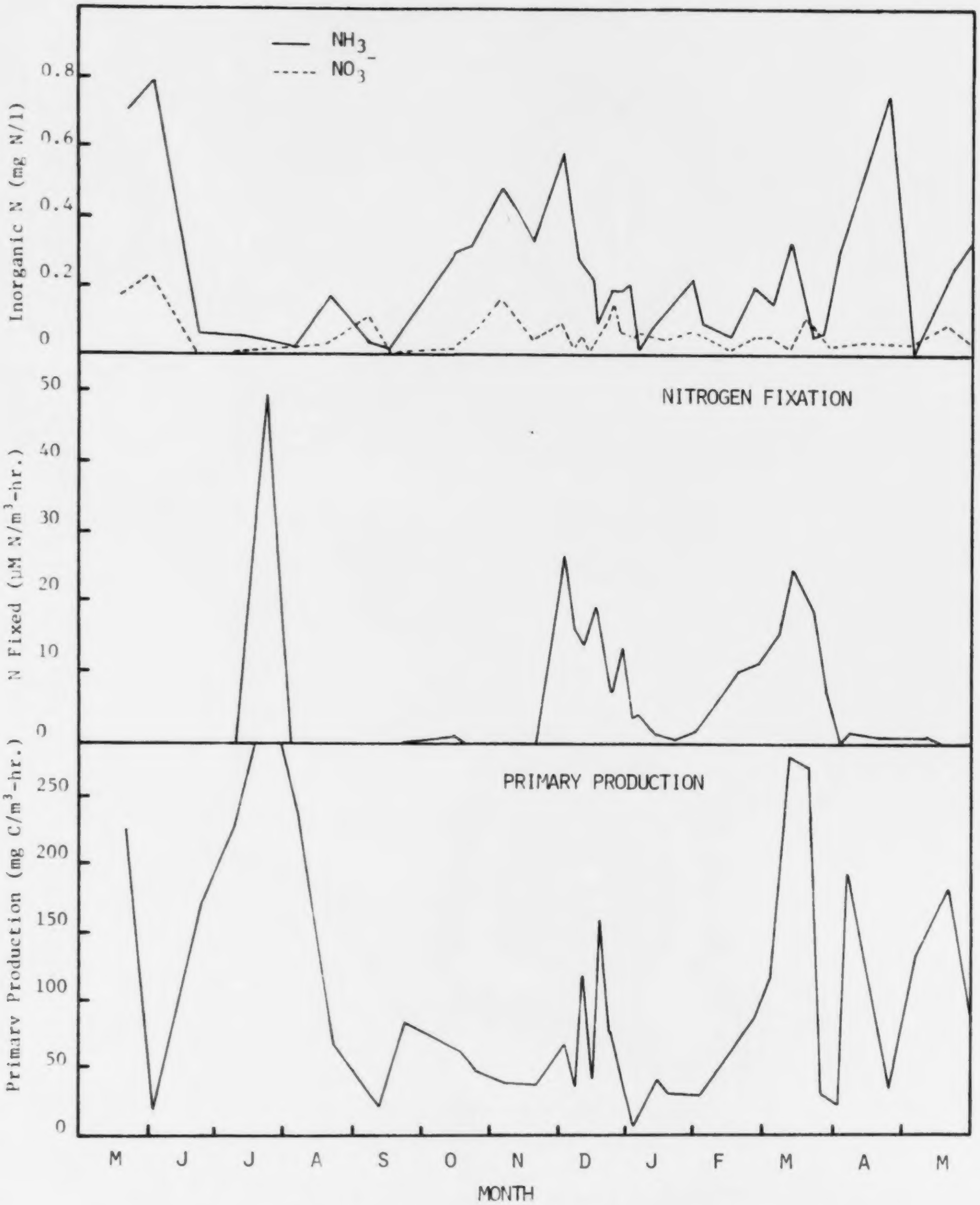

FIGURE 6. INORGANIC NITROGEN CONCENTRATIONS, NITROGEN FIXATION, AND PRIMARY PRODUCTION IN NEWNAN'S LAKE, FLORIDA FROM MAY, 1969 TO MAY, 1970

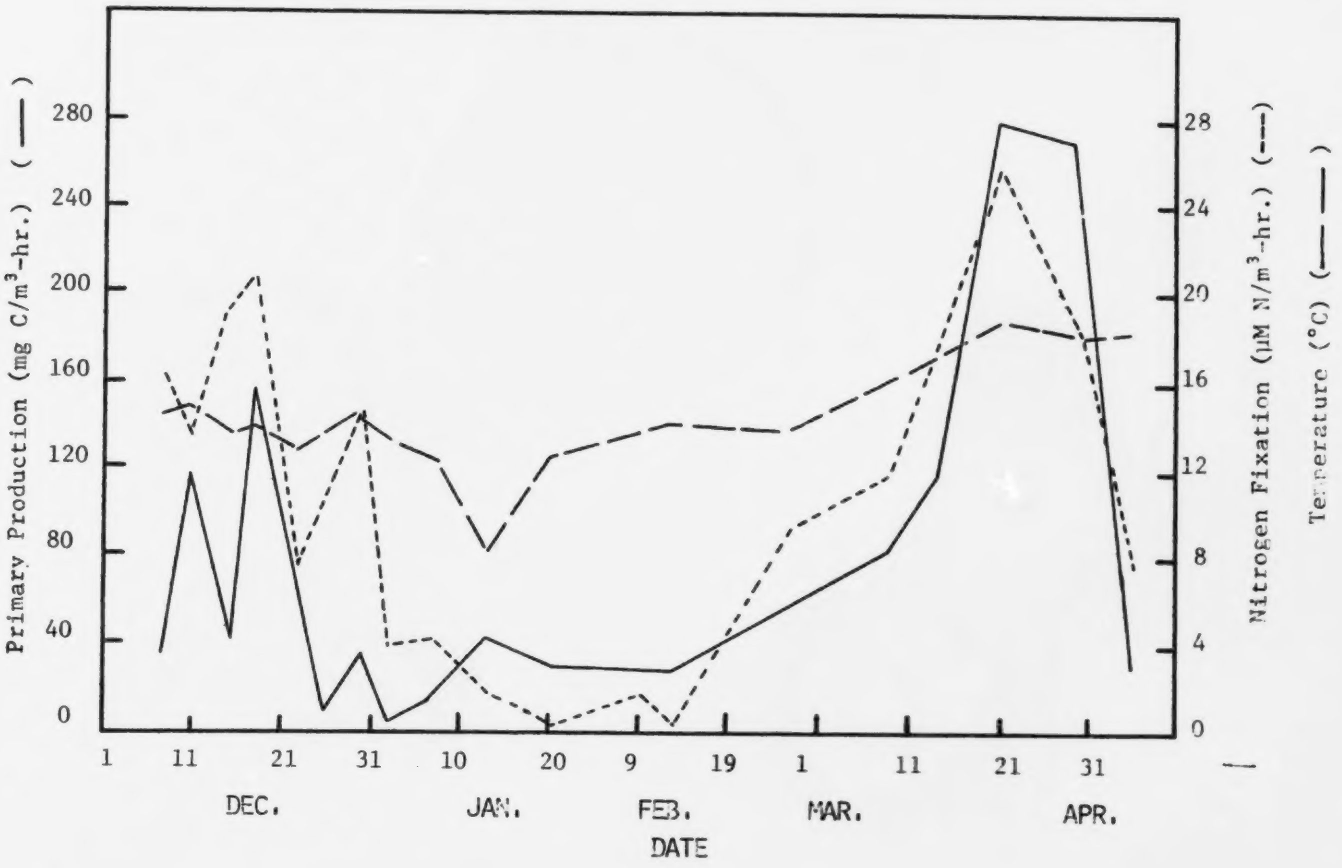

FIGURE 7. TEMPERATURE, PRIMARY PRODUCTION AND NITROGEN FIXATION IN NEWNAN'S LAKE DURING WINTER (1969-1970) *APHANIZOMENON* BLOOM.

Table 12. Areal Variations in Physical and Biogenic Parameters in Newnan's Lake, 19 April, 1969

	Station										Mean and 95%	
Parameter[1]	1	2	3	4	5	6	7	8	9	10	Confidence Interval	Coefficient of Variation (%)
Sfc. Temp.	22.0	22.4	22.4	22.5	22.3	22.3	22.5	22.0	22.1	22.4	22.3	--
Secchi Disc	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	--
Tot. depth	1.35	1.5	2.0	2.0	2.6	1.8	1.8	1.8	1.8	1.8	--	--
D. O.	6.40	6.00	5.80	5.80	5.90	5.70	4.50	5.90	5.50	6.50	5.8 ± 1.0	28.3
pH	7.05	7.05	6.90	7.05	7.00	7.00	6.95	7.00	7.00	7.05	7.00 (median)	--
Alk.	10.4	10.7	9.95	10.0	10.0	10.1	9.71	10.1	10.3	10.4	10.2 ± 0.5	8.3
TON	1.51	1.64	1.55	1.68	1.40	1.77	1.88	1.48	1.46	1.44	1.58 ± 0.29	30.4
NH_3-N	0.61	0.71	0.78	0.82	0.84	0.76	1.06	0.77	0.94	0.92	0.82 ± 0.24	47.6
NO_3^--N	0.07	0.03	0.03	0.02	0.02	0.02	0.02	0.03	0.04	0.00	0.028 ± 0.003	17.9
o-PO_4-P	0.023	0.010	0.010	0.010	0.012	0.010	0.012	0.019	0.025	0.020	0.015 ± 0.011	118
t-PO_4-P	0.167	0.144	0.156	0.184	0.152	0.108	0.188	0.160	0.140	0.160	0.162 ± 0.029	28.7
Prim. Prod.	20.8	32.4	43.5	71.0	41.7	92.0	51.1	38.8	16.5	51.1	46.0	--

---

[1] Temp. in °C; Secchi disc visibility and total depth in meters; pH = -log (H^+); alkalinity in mg/l as $CaCO_3$; primary production in mg C/m^3-hr; all other values in mg/l.

shore for processing. Primary production and nitrogen fixation samples were then returned to the lake for incubation at a near shore station. Although nitrogen fixation was not occurring in the lake on this day, the data are nevertheless useful in describing the variability in biological conditions. The variations in biogenic parameters was much larger than in the physical parameters and in pH and alkalinity. Primary production had by far the greatest variability - from 21 to 92 mg C/m^3-hr. Newnan's Lake obviously cannot be considered homogeneous, and any one sampling station may not be representative of the entire lake at a given time. In order to obtain representative data and still keep the sampling and analyses within reasonable limits, it was decided to composite 6-8 stations scattered at random around the lake into one sample for the routine bi-weekly analyses. Thus the data in Figure 6 represent determination on a composite lake water sample and are felt to reflect closely the average conditions in the lake at a given time. Because Bivin's Arm is a much smaller and presumably better mixed lake, it was not felt necessary to composite so many stations. Consequently the data in Figure 5 represent composite samples from three stations and 3 depths.

A second areal variation study was conducted on Newnan's Lake December 8, 1969, at the onset of the Aphanizomenon bloom. Again 10 stations were sampled and nitrogen fixation was found at each station. Figure 8 shows the distribution of primary production and nitrogen fixation rates at the 10 stations; the former varied in a much smaller range on this date than in the April study. Nitrogen fixation was much more variable than primary production and the two rates do not seem correlated. Table 13 presents depth variations found at two stations and summarizes the variations found in nitrogen fixation, primary production and chlorophyll. It is interesting to note that areal variations with the latter biomass measure were somewhat smaller than those of primary production. The large vertical differences in these parameters in so shallow a water column are probably the result of self-shading. Note that chlorophyll decreased less than did the activity parameters - primary production and nitrogen fixation which are light dependent phenomena.

Diel variations in Newnan's Lake were measured on three occasions: April 21, 1969, December 16, 1969 and April 20, 1971. Nitrogen fixation was found in the lake only in the December study. The April, 1971, study apparently just missed the annual Aphanizomenon bloom, which was quite evident in the lake a few days earlier. Figure 9 shows the time course of primary production, nitrogen fixation and light intensity during the December 16, 1969, study. Sampling began at 7:00 A. M. and continued at about one and a half hour intervals until dark. Primary production reached a maximum value at mid-morning and decreased markedly during the afternoon, at least partly because an increased cloud cover reduced light intensity. Peak nitrogen fixation lagged behind photosynthesis and occurred in early afternoon. Although sampling was not continued after dark the data suggest that nitrogen fixation is limited to daylight hours or at least occurs at much lower rates during the night. Figure 10 presents some results of a more detailed diel study conducted in April, 1971. Unfortunately the lake was not fixing nitrogen at this

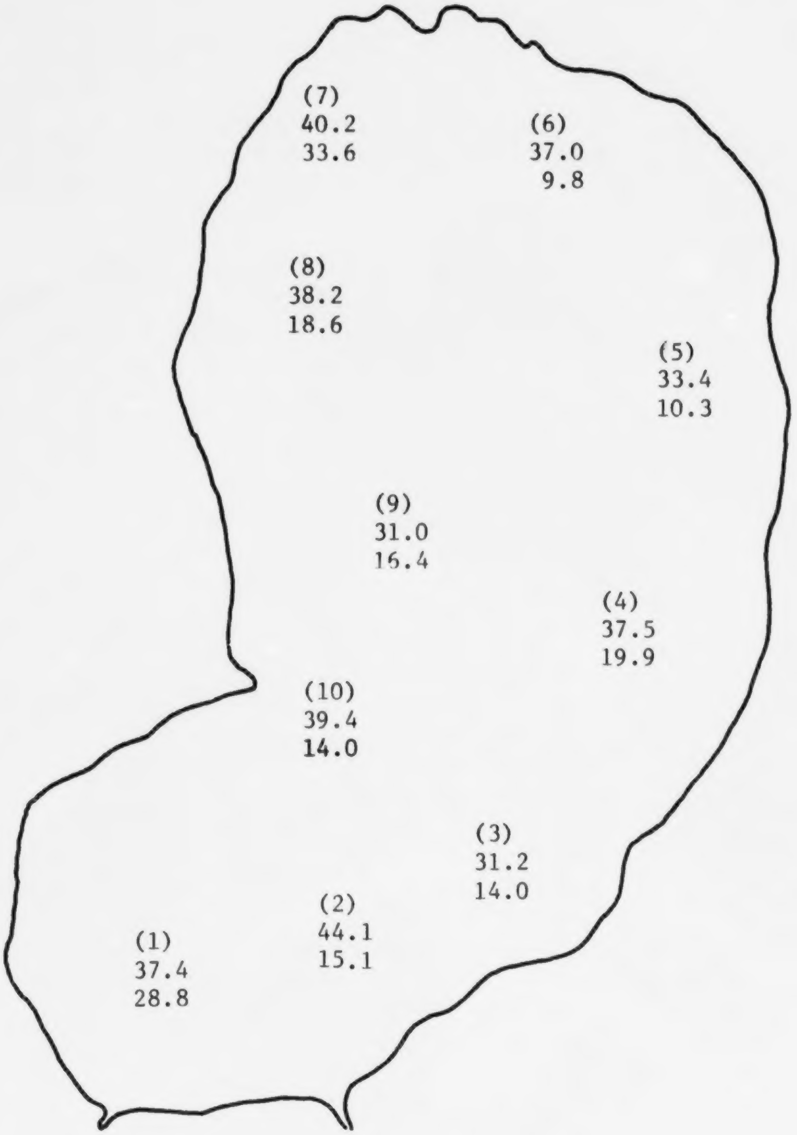
(7)
40.2
33.6
(6)
37.0
9.8
(8)
38.2
18.6
(5)
33.4
10.3
(9)
31.0
16.4
(4)
37.5
19.9
(10)
39.4
14.0
(3)
31.2
14.0
(2)
44.1
15.1
(1)
37.4
28.8

FIGURE 8. AREAL VARIATIONS IN NEWNAN'S LAKE, DECEMBER 8, 1969. STATION NUMBERS IN PARENTHESES; UPPER VALUE: PRIMARY PRODUCTION IN mg C/m^3-hr; LOWER VALUE: NITROGEN FIXATION IN μM N/m^3-hr.

Table 13. Vertical and Areal Variations in Nitrogen Fixation and Related Parameters in Newnan's Lake, 8 December 1969

Depth m.	Primary Production mg C/m^3-hr	Nitrogen Fixation nM N/l-hr	Chlor. a. mg/m^3	Temp. °C
		Station 4		
Sfc	38	20	70	13.8
1	19	14	69	13.3
2	20	10	64	13.3
		Station 8		
Sfc	38	19	70	14.8
2	19	18	71	13.6
4	17	6	64	13.3
		Summary of Areal Variations[1]		
Mean	36.3	18.1	67.4	--
Range	31.0-44.1	9.8-33.6	60.7-75.1	--

[1] Means of ten stations, eight of which were samples composited with depth and two of which (stations 4 and 8 above) were samples taken at discrete intervals. Mean values over depth for these two stations were used for calculation of the areal means.

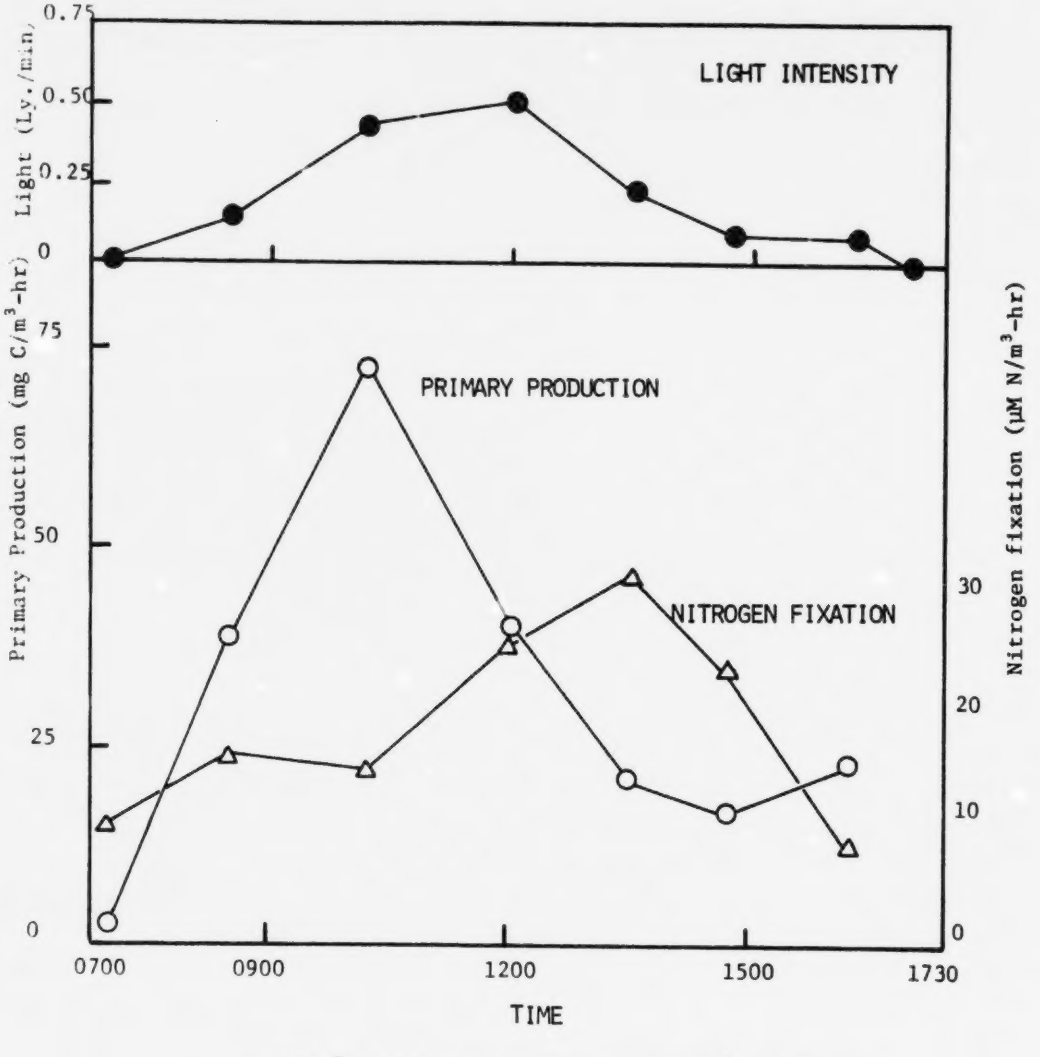

FIGURE 9. DIEL VARIATIONS IN NEWNAN'S LAKE, DECEMBER 16, 1969.

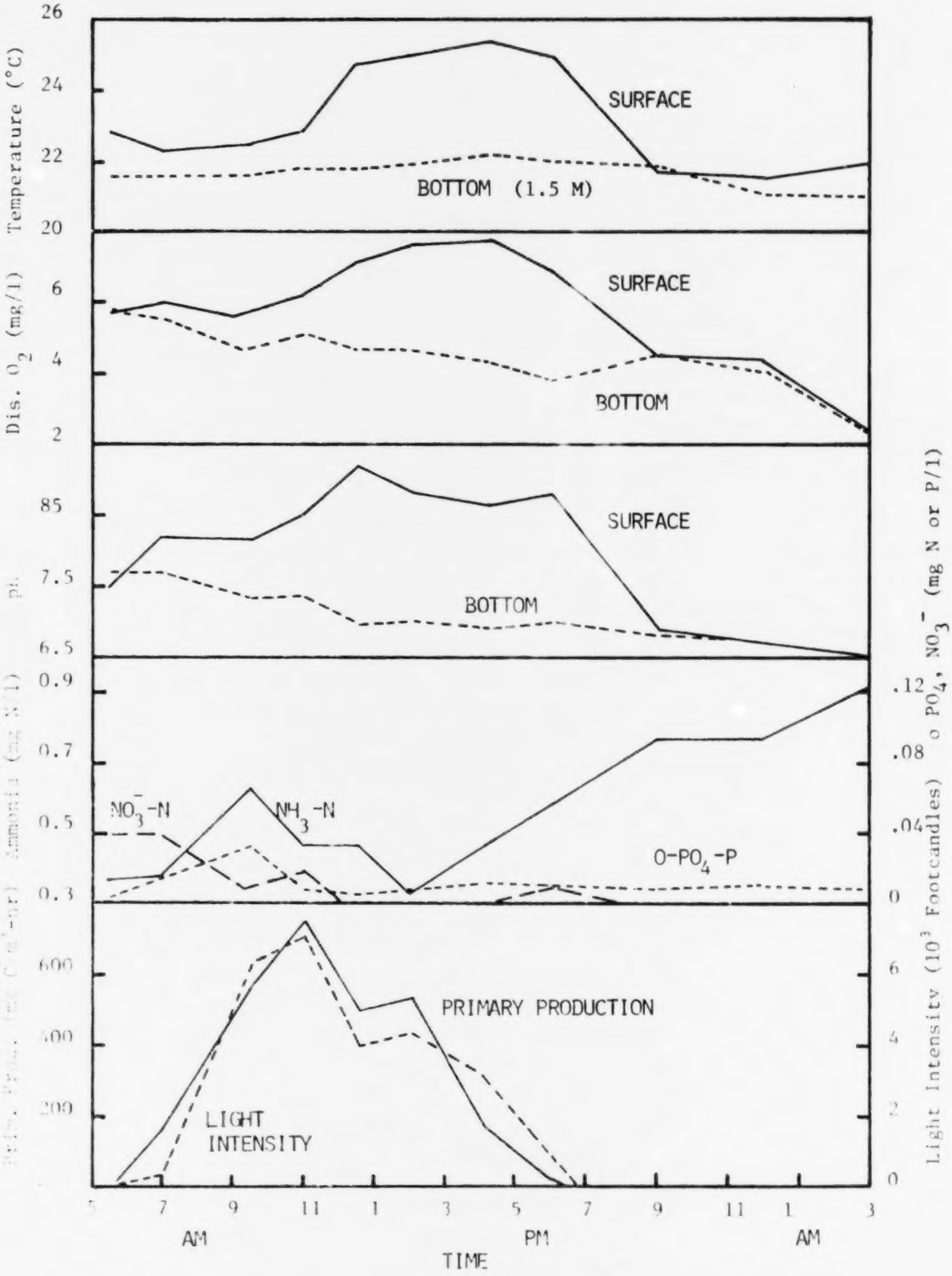

FIGURE 10. VARIATIONS IN SELECTED PHYSICAL AND BIOGENIC PARAMETERS DURING DIEL STUDY IN NEWNAN'S LAKE, APRIL, 1971.

time but the data are interesting in illustrating the large changes in biogenic parameters that can occur on a daily basis in eutrophic lakes. The lake was very calm during this study, and a slight, temporary temperature stratification occurred during the day. Dissolved oxygen values rose during daylight hours but fell much below saturation after nightfall. The small decline in oxygen after several hours of sunlight seems to be a case of photorespiration, as recently described by Odum (1971). The total change in oxygen content during the day was dramatic; considering the calm conditions most of the increase would appear to arise from photosynthesis rather than gas transfer from the atmosphere. Nutrients declined somewhat irregularly during the daylight hours, but the ratios of carbon, nitrogen and phosphorus net assimilation during the period of peak photosynthesis can be approximated from the data in Figure 10. During the period 9:30 A. M. to 4:15 P. M. photosynthesis resulted in a net increase of 2.1 mgO_2/l in the surface waters. Before and after this period photosynthesis was less than or about equal to respiration as the dissolved oxygen values indicate. During the period of maximum photo synthesis a net decrease of 0.16 mg NH_3-N/l and 0.022 mg ortho PO_4-P/l occurred in the surface water. Converting the oxygen increase to equivalent carbon fixed indicates a net of about 0.75 mgC/l assimilated. On a molecular basis then these values indicate net C:N:P uptake ratios of 100:18:1·1 on a molar basis, which ratios are quite similar to the average C:N:P composition ratios reported for algal cells (i.e. 106:16:1).

The rather large diel changes in biogenic parameters reflect the dynamic nature of nutrient cycling especially in eutrophic lakes. These changes also imply that routine sampling for seasonal or other long term studies should be conducted at approximately the same time of day. Otherwise changes resulting from diel cycles may erroneously be thought of as reflecting longer term variations.

The severity and duration of the annual *Aphanizomenon* bloom in Newnan's Lake is apparently quite variable. In winter 1968-1969 the bloom occurred from late December to mid February, and at its height was more intense than during the following year. Two samples taken near shore on January 20, 1969, were thick with clumps of *Aphanizomenon*, and yielded fixation rates of 70 and 163 nM C_2H_4/l.-hr., which values are considerably in excess of the maximum values for the winter 1969-1970 bloom. On the other hand massive concentrations of the algae near shore frequently result from sustained winds, so the above values are not necessarily representative of fixation rates in the entire lake during the January, 1969, bloom. The bloom of *Aphanizomenon* in 1971 appeared much later (late March) and was of much shorter duration than in the previous year. These differences are no doubt associated with differences in weather conditions (rainfall, temperature) from year to year. However this qualitatively simple explanation would be very difficult to verify in a quantitative manner, and attempts to correlate the bloom occurrence and duration with climate data collected during this study were not fruitful. Perhaps a detailed analysis of past weather records and bloom conditions (if they were available) could yield an empirical explanation, but that is beyond the scope of this project. While the details and absolute amounts of fixation in the lakes may vary yearly, the pattern seems qualitatively similar from year to year. Thus the data presented here are regarded as typical, and in this sense can be utilized for more general conclusions on the importance of fixation in Florida lakes.

An obvious but unanswered question about the patterns of fixation in these two lakes concerns its occurrence primarily as a winter phenomenon in one lake but as a summer phenomenon in the other lake in spite their proximity and essentially identical climate. The large volume of data (Appendix B) collected during the Aphanizomenon bloom in Newnan's Lake during winter, 1969-1970 has been scrutinized and subjected to statistical analysis in order to elicit trends and potential causal factors for the appearance and eventual demise of the bloom. However there does not appear to be any clear answer, and bloom formation and disappearance (hence also the occurrence of nitrogen fixation) may be controlled by a subtle interaction of physical and chemical factors, or by biological causes that were not measured. Several possibilities are suggested: initiation of the bloom by temperature changes (e.g. an increase in temperature following a cold period during which Microcystis and the other algae present in abundance during the bulk of the year are eliminated); changes in light intensity or in photoperiod, or perhaps a combination of temperature and light conditions are necessary. There do not appear to be any substantial chemical changes in the lake prior to bloom onset, but the possibility of a biologically induced change in an organic growth factor or in a required trace metal such as iron or molybdenum should not be dismissed. Possible reasons for bloom disappearance are even more numerous. This strain of algae may have rather narrow temperature requirements, and the pulse shown in Figure 7 can be explained on this basis. When the lake temperature declined because of a severe cold spell in early January, 1970 (Figure 7), the bloom rapidly died out. Only after the temperature rose above 14°C in late February were the algae able to reach dense bloom conditions again, but once the temperature rose above about 20° in late March the algae again died out or alternatively were no longer able to compete successfully against other forms. In support of this hypothesis a multiple regression analysis (Table 14) of primary production vs. temperature, light, ammonia and ortho phosphate in the lake during this bloom found temperature to be the most important variable (statistically). In fact using a multiplicative model (logarithmic transformation) temperature alone explained nearly as much of the variance as did all four variables combined. However temperature alone is probably too simple an explanation and there are discrepancies in the argument. There is no information in the literature suggesting that Aphanizomenon has such a narrow temperature tolerance (Hammer, 1964). In fact blooms of this algae in temperate lakes (e.g. in Wisconsin) are common in mid and late summer when water temperatures are between 20 and 25°C. Further, the drop in Newnan's Lake temperature in January 1970 was also associated with large quantities of rain which raised the lake level as much as three feet. Consequently the Aphanizomenon bloom was both diluted and transported out of the lake in the unusually high out-flows generated by the rain. Alternative explanations for the blooms demise also exist, including the possibility of nutrient exhaustion (probably a trace nutrient since inorganic phosphate remained low but not depleted during the entire bloom

Table 14. Limiting Factors for Primary Production in Newnan's Lake, Florida During Aphanizomenon Bloom

Multiple Regression Analysis

Dependent Variable: Primary Production
Independent Variables: Light, Temperature, NH_3-N, Ortho-PO_4-P

1. Additive Model:

$$Y = a_1X_1 + a_2X_2 + a_3X_3 + a_4X_4$$

$$PP = 4.76(\text{Temp}) + 4.32(\text{o-}PO_4) - 6.05(\text{Light}) - 69.4(NH_3\text{-N})$$

Var. Entered	R^{2*}	Increase In R^2
Temp.	0.6579	0.6579
o-PO_4	0.6812	0.0233
Light	0.6953	0.0141
NH_3	0.6995	0.0042

* R^2 - measure of portion of variance accounted for by regression

2. Multiplicative Model:

$$Y = X_1^{b_1} X_2^{b_2} X_3^{b_3} X_4^{b_4}$$

$$\ln Y = b_1 \ln X_1 + b_2 \ln X_2 + b_3 \ln X_3 + b_4 \ln X_4$$

$$PP = 1.65(\text{Temp}) - 0.26(\text{Light}) - 0.14(\text{o-}PO_4) + 0.07(NH_3\text{-N})$$

Var. Entered	R^2	Increase In R^2
Temp.	0.9416	0.9416
Light	0.9425	0.0008
o-PO_4	0.9432	0.0007
NH_3-N	0.9433	0.0001

and nitrogen would not seem a limiting element to a nitrogen fixing bloom). Viral attack is currently a popular explanation among aquatic scientists for otherwise unexplainable bloom disappearances, as is autointoxication (i.e. synthesis of a substance by the algae which causes their own death), but neither possibility has ever been proved to cause bloom death in the environment. In brief, the complexities of algal bloom cycles are still beyond simple explanation and comprehension. While many causes can be conjectured, unfortunately they remain only that.

Seasonal patterns of nitrogen fixation, nutrient concentrations, primary production and other biogenic parameters are poorly defined in these Florida lakes in comparison with classic cycles reported for temperate lakes. Rather, production, nutrients and algal populations are characterized by a bloom-crash cycle throughout the year. Pronounced differences were noted between sampling dates even when sampling was done as frequently as twice a week. The large measured changes in chemical and biological parameters from one sampling to the next were matched by obvious visual changes in the lakes, and many of the rapid fluctuations can be ascribed to rapidly changing weather conditions.

The diel variations coupled with the large oscillations in fixation from one sampling date to the next obviate accurate assessment of the total quantities of nitrogen fixed in the lakes during a year. However, the data in Figure 5 and 6 should permit at least a rough estimate. Assuming nitrogen fixation is primarily a daylight phenomenon, and during periods of fixation it occurs in the lakes for about 8 hours a day at the rates shown in Figures 5 and 6, the nitrogen fixed in the 2 lakes over the year study was calculated, and the results are summarized in Table 15. While the total N fixed is large in both lakes, fixation represents a relatively small contribution to the total nitrogen budgets previously calculated for these lakes by Shannon (1970) (see Section V). Based on the survey of Florida lakes described earlier, it is unlikely that nitrogen fixation would play a substantially greater role in the nutrient budgets of other Florida lakes. Hence, nitrogen fixation in lakes seems to be of greater significance in an ecological sense than as a nutrient source.

Table 15. Contributions of Fixation to the Nitrogen Budgets of Newnan's Lake and Bivin's Arm

	N Fixed	External N Supply g/m^3yr	Total N Supply	Percent of Total Supplied by Fixation
Newnan's Lake	0.15	2.60	2.75	5.5
Bivin's Arm	0.52	6.86	7.38	7.0

[1] Calculated from watershed characteristics (land use and population patterns) by Shannon and Brezonik (1971a) - see Section V.

SECTION VII

NITROGEN FIXATION AS AN IN SITU NITROGEN SOURCE FOR NATURAL WATERS

II. BACTERIAL FIXATION IN LAKES AND SEDIMENTS

While numerous studies have identified and isolated nitrogen fixing bacteria from lakes and marine waters (see Section IV), most previous investigators have considered heterotrophic (bacterial) fixation to be of little consequence. A lack of oxidizable carbohydrate has usually been cited as the limiting factor for heterotrophic fixation in both soils and natural waters (Stewart, 1969). Various reports (see Stewart , 1966, for a review) indicate that heterotrophic fixation under laboratory (pure culture) conditions is highly inefficient in terms of nitrogen fixed per unit of carbon oxidized. However it is always dangerous to extrapolate highly artificial laboratory results to *in situ* conditions. Studies conducted as a part of this project indicate that heterotrophic (bacterial) fixation can be important in certain anoxic lacustrine environments as well as in sediments. In addition the ability of photosynthetic bacteria to fix nitrogen is well known (Stewart, 1966) and while these organisms occupy a rather special niche (i.e. they require anoxic conditions and low light intensity) and are not generally abundant in the environment, they may fix substantial amounts of nitrogen in favorable habitats. For example, Stewart (1969) correlated significant uptake of $^{15}N_2$ at a depth of 7 m. in a Norwegian fjord with the presence of the photosynthetic bacterium *Pelodictyon*. Trüper and Genovese (1968) have reported high concentrations of photosynthetic bacteria occurring at intermediate depths in certain Italian lakes.

The first evidence for bacterial nitrogen fixation in lakes was reported by Brezonik and Harper (1969) in work supported by this project. They found fixation in two dystrophic lakes chosen for their extensive anoxic environments. Lake Mary, Wisconsin, is a colored meromictic lake, permanently anoxic below 5 meters (maximum depth = 21 meters). Lake Mize, Florida, is a dystrophic, highly colored lake with a maximum depth around 25 meters and a surface area of 0.9 ha. The morphometry of Lake Mize (Figure 13) does not promote good vertical circulation, but the lake is monomictic with a circulation period from October or November to the end of February or early March depending on weather conditions. Oxygen is lost during the long period of stratification, and the lake is anoxic below about 3 to 5 meters from June to November.

Lake Mary was sampled June 28, 1968, and Lake Mize on July 11, and August 21, 1968. Profiles of some pertinent lake conditions are shown in Figures 12 and 13 for Lakes Mary and Mize, respectively. Rates of acetylene reduction at various depths in the two lakes appear in

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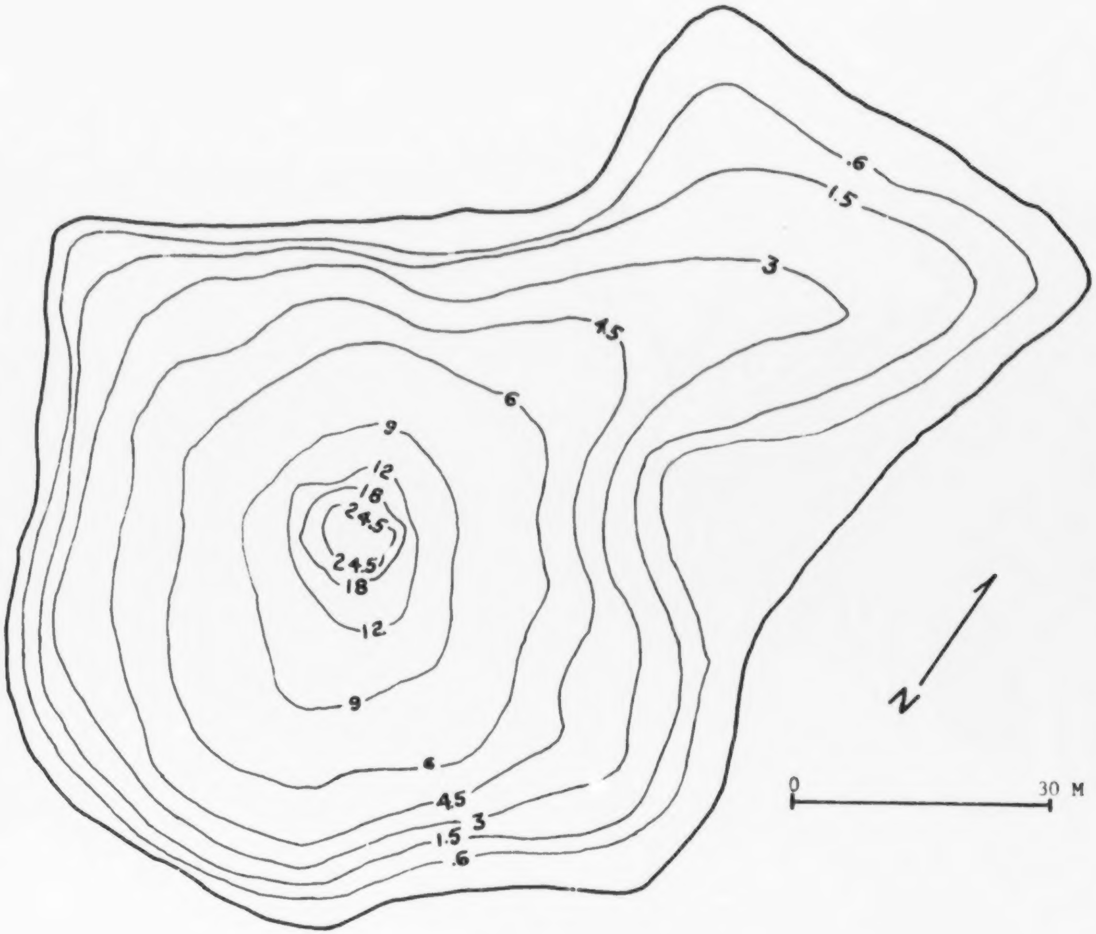
.6
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30 M

FIGURE 11

BATHYMETRIC MAP OF LAKE MIZE, ALACHUA COUNTY, FLORIDA. CONTOURS IN METERS. MAP COURTESY OF F. G. NORDLIE.

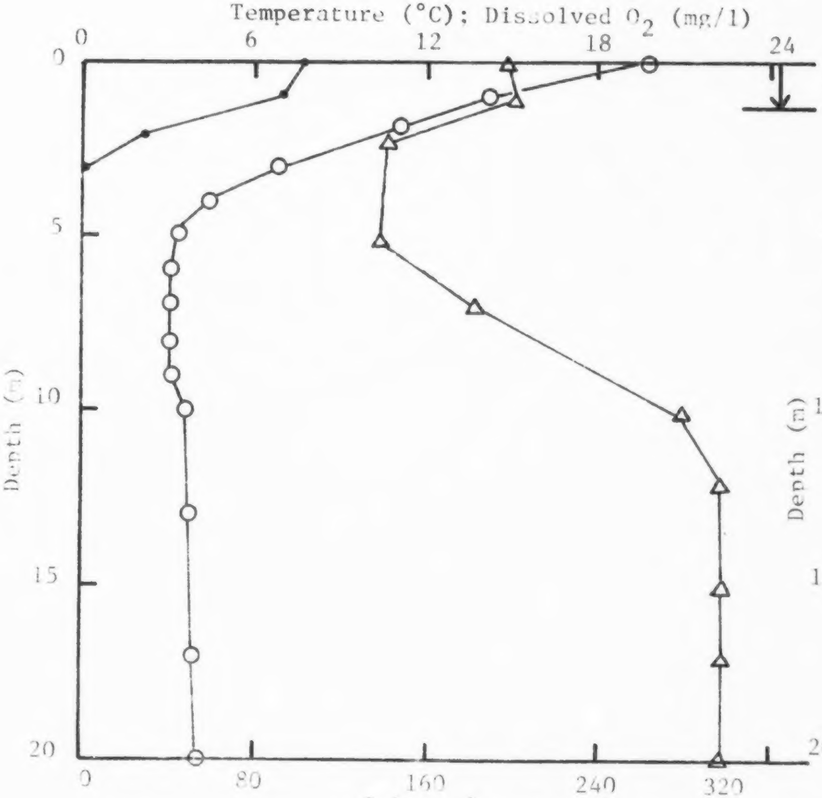

FIGURE 12. DEPTH PROFILES FOR TEMPERATURE (o), DISSOLVED OXYGEN (●) AND COLOR (Δ) IN LAKE MARY, WISCONSIN, JUNE 28, 1968. ARROW INDICATES DEPTH OF SECCHI DISC VISIBILITY.

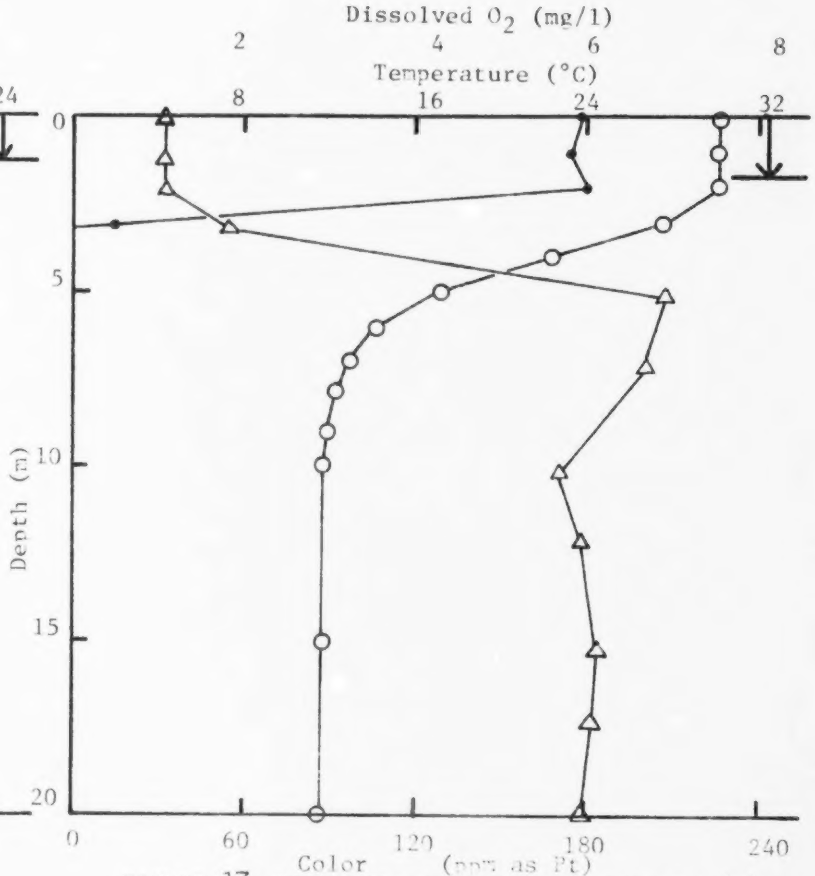

FIGURE 13. DEPTH PROFILES FOR TEMPERATURE (o), DISSOLVED OXYGEN (●) AND COLOR (Δ) IN LAKE MIZE, AUGUST 1, 1968. ARROW INDICATES DEPTH OF SECCHI DISC VISIBILITY.

Table 16. Rates measured in this initial study were generally low compared with fixation rates found in some eutrophic surface waters containing blue-green algae. The highest rate in Lake Mary occurred near the bottom, but in Lake Mize maximum activity was noted at a depth of 15 m.

A detailed study of nitrogen fixation in Lake Mize was conducted during 1969 and 1970 to determine the extent and significance of bacterial fixation in the lake (Keirn and Brezonik, 1971). Table 17 presents a summary of the general chemical characteristics of Lake Mize. The lake has a high but variable color, which is evidently leached from pine needle litter in the drainage basin, and has typical characteristics of dystrophy, i.e. low values of pH, calcium, and primary productivity. Figures 14 and 15 present temperature and dissolved oxygen profiles of the lake for 1969 and 1970. In 1969 the lake was only slightly thermally stratified in February but a marked depletion of dissolved oxygen was found in the hypolimnion. By April anoxic conditions had developed below 7 meters and by July no oxygen was present below 3 meters. Stratification began somewhat later in 1970, but dissolved oxygen was partially depleted in the bottom waters during February. Anoxic conditions occurred below 5 meters by early April and below 3 meters by early June; it is also interesting to note that dissolved oxygen values were low even in surface waters during the summer of 1970.

Conditions in Lake Mize have changed during the last several years as a result of an increased nutrient loading. In fall of 1968 an enclosure housing about 50 ducks was placed at the north shore of the lake. In response to enrichment, a lush growth of emergent grass occurred along the previously nearly bare shoreline and the rate of primary production increased markedly. Rates ranging from 1.9 to 10.4 mg. C/m.^{3}-hr. were found prior to 1970, but samples taken in April and June, 1970, yielded rates of 42.3 and 36.2 mg. C/m.^{3}-hr., respectively. Concentrations of nitrogen and phosphorus also increased substantially from 1968 to 1970 (Table 18), presumably reflecting duck enrichment. Low concentrations of ammonia occurred throughout the water column in spring of 1969 and an inverse clinograde distribution occurred in summer. By September 1969 epilimnetic ammonia concentrations surpassed those in the bottom water. Concentrations were higher throughout 1970 and generally showed more complex profiles. Total organic nitrogen was rather uniform throughout the water column during the two years, and nitrate levels remained low (typically $<$ 0.05 mg. N/l.) but somewhat variable during the study.

Nitrogen fixation as measured by the acetylene reduction method was found during summer stratification in 1969 and 1970. Figure 16 illustrates the relationship of fixation rate to depth during the periods of fixation; it is noted that fixation occurred only in the hypolimnion during both years. Rates were highest during the summer of 1969, and the depth at which peak fixation occurred became greater over the course of this summer. This tendency was not found during 1970 when fixation started later than 1969 and ended sooner. Why a

Table 16. Acetylene Reduction in Lakes Mary and Mize

Lake Mary-June 28, 1968				Lake Mize-July 11, 1968				Lake Mize-August 21, 1968			
Depth [1] meters	TKN[2] mg/1	nM C_2H_4/ 1-hr.	ng N/ [3] 1-hr.	Depth[1] meters	TON[2] mg/1	nM G_2H_4/ 1-hr.	ng N/ [3] 1-hr.	Depth [1] meters	TKN [2] mg/1	nM C_2H_4/ 1-hr	ng N/ [3] 1-hr
0 a	0.93	0.54	5.0	0 a	0.56	17.8	166	0 a	0.38	0	0
2 b	3.1	0.36	3.3	1.5 a	0.56	14.3	133	2 a	0.60	0	0
5 b	1.8	0.89	8.3	3.0 a	0.63	12.5	117	5 a	0.69	0	0
7 b	2.9	0.98	9.2	6.1 a	0.65	11.6	108	7 b	0.73	2.98	27.8
10 b	2.5	1.70	15.8	6.1 a	0.65	8.9	83	10 b	0.77	3.28	30.6
15 b	3.3	1.16	10.8	9.1 b	0.70	33.0	308	15 b	1.29	8.93	83.3
20 b	4.5	5.00	46.6	18.3 b	0.69	10.7	100	20 b	0.66	2.68	25.0

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[1] Samples were purged with oxygen-argon mixture (a) or helium (b) before incubation.

[2] TKN = total Kjeldahl nitrogen; TON = total organic nitrogen, both in mg N/1.

[3] Calculated from nanomoles of ethylene produced per liter-hour assuming theoretical ratio of 1.5 moles ethylene produced per mole of ammonia fixed.

Table 17. Chemical Characteristics of Lake Mize, Florida[1]

pH	5.03
Acidity (mg $CaCO_3$/l)	29.4
Alkalinity (mg $CaCO_3$/l)	1.7
Specific conductance (μmho cm^{-1})	52.9
Color (mg Pt/l at pH 8.3)	434
Turbidity (JTU)	1.4
Chloride (mg/l)	10.3
Sulfate (mg/l)	5.0
Na (mg/l)	6.7
K (mg/l)	0.32
Mg (mg/l)	0.94
Ca (mg/l)	3.4
Fe (mg/l)	2.4
Mn (μg/l)	19
F (mg/l)	0.02
SiO_2 (mg/l)	3.19
COD (mg/l)	69
Total solids (mg/l)	77
Suspended solids (mg/l)	2

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[1] Average of Data collected from June 1968 to June 1970 (Shannon 1970).

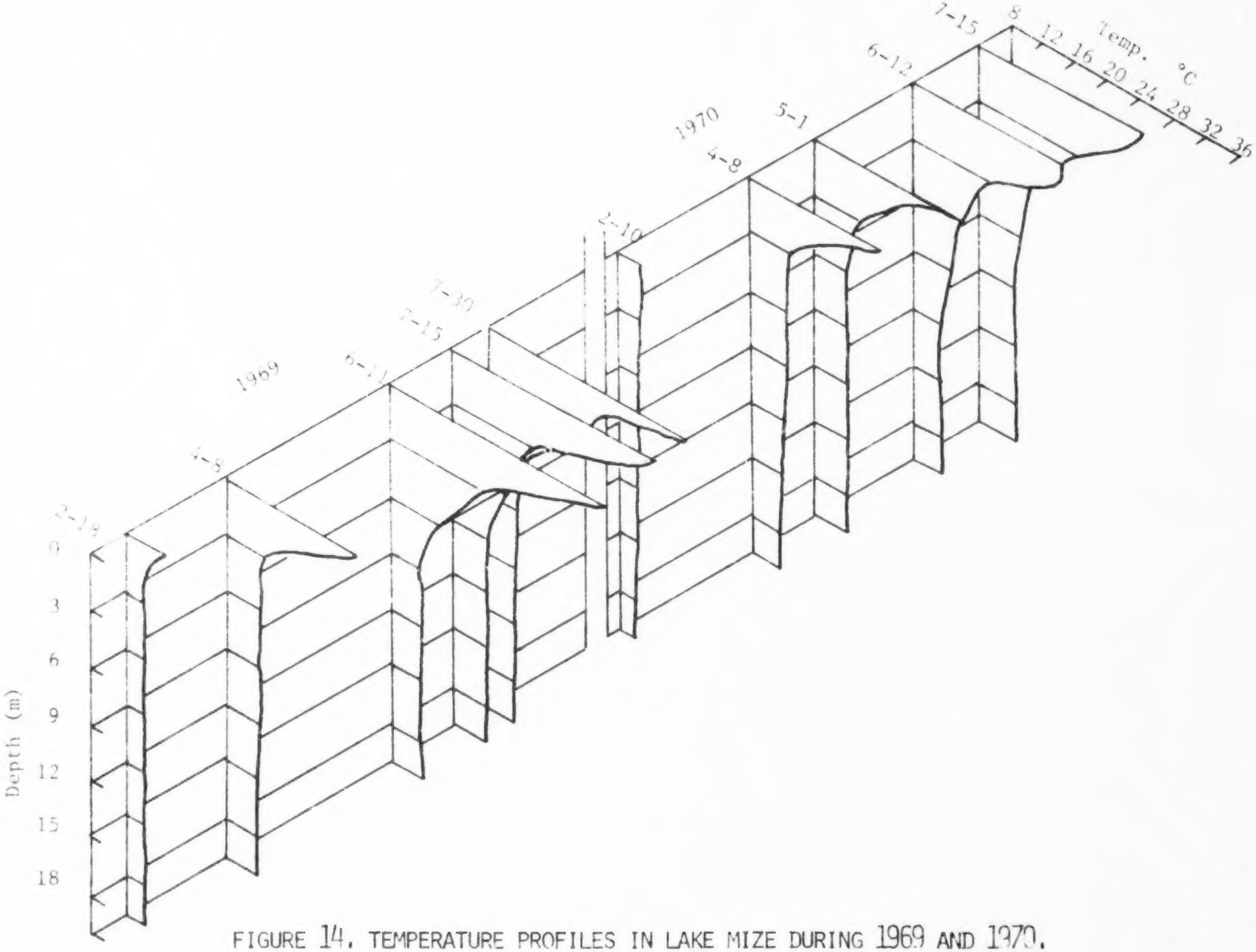

FIGURE 14. TEMPERATURE PROFILES IN LAKE MIZE DURING 1969 AND 1970.

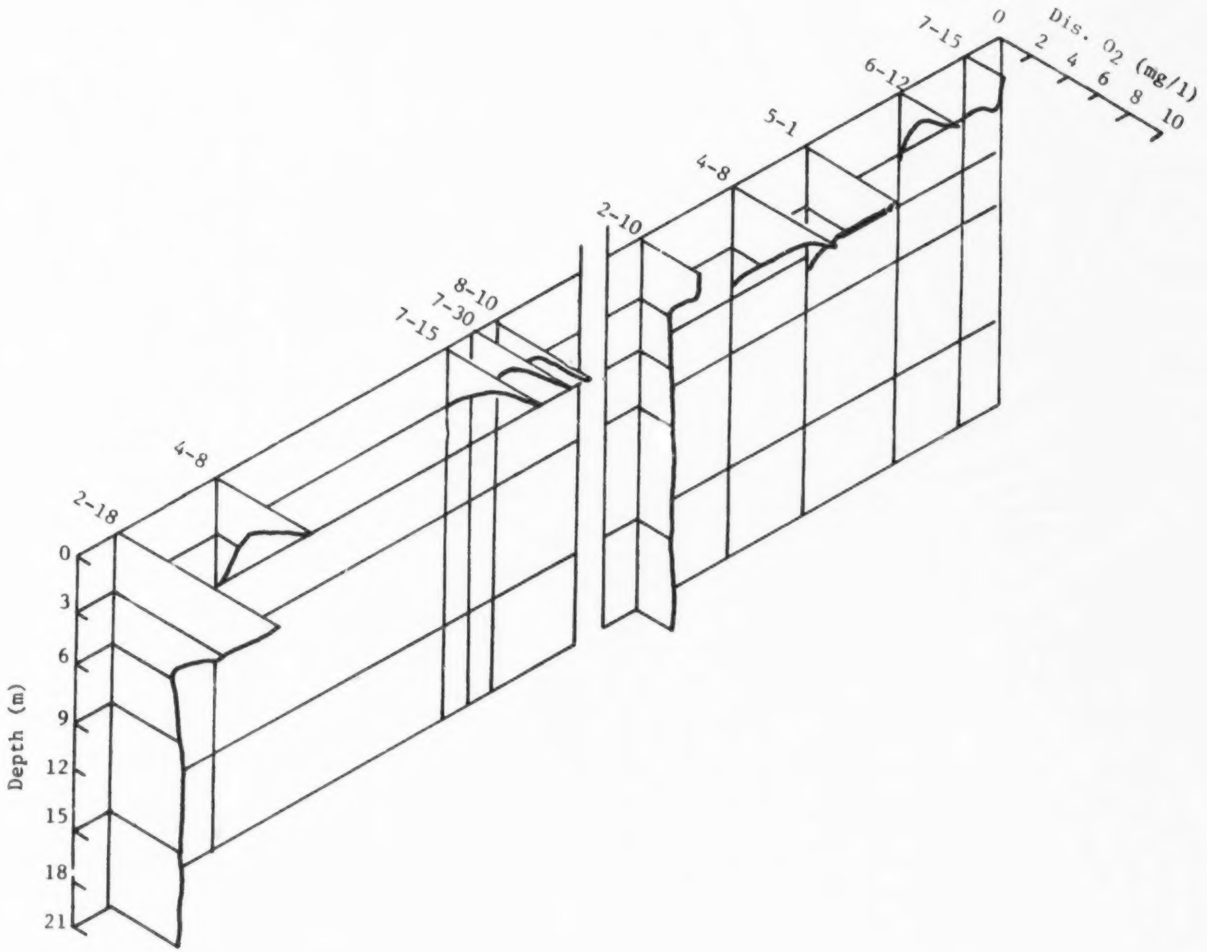

FIGURE 15. DISSOLVED OXYGEN PROFILES IN LAKE MIZE DURING 1969 AND 1970.

Table 18. Changes in Nutrient Levels in Lake Mize, 1968-1970[1]

	August, 1968		June, 1969		June, 1970	
Parameter[2]	Top	Bottom	Top	Bottom	Top	Bottom
Total organic N	0.25	0.63	0.82	0.66	1.35	1.02
Ammonia-N	0.20	0.40	0.03	0.07	0.50	0.28
Ortho phosphate-P	0.000	0.063	0.018	0.030	0.058	0.10
Total phosphate-P	0.005	0.063	0.087	0.059	0.15	0.19

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[1] Values represent means of top 3 meters and bottom 15 meters, or approximately the mean epilimnetic and hypolimnetic concentrations.

[2] Values in mg/l as N or P.

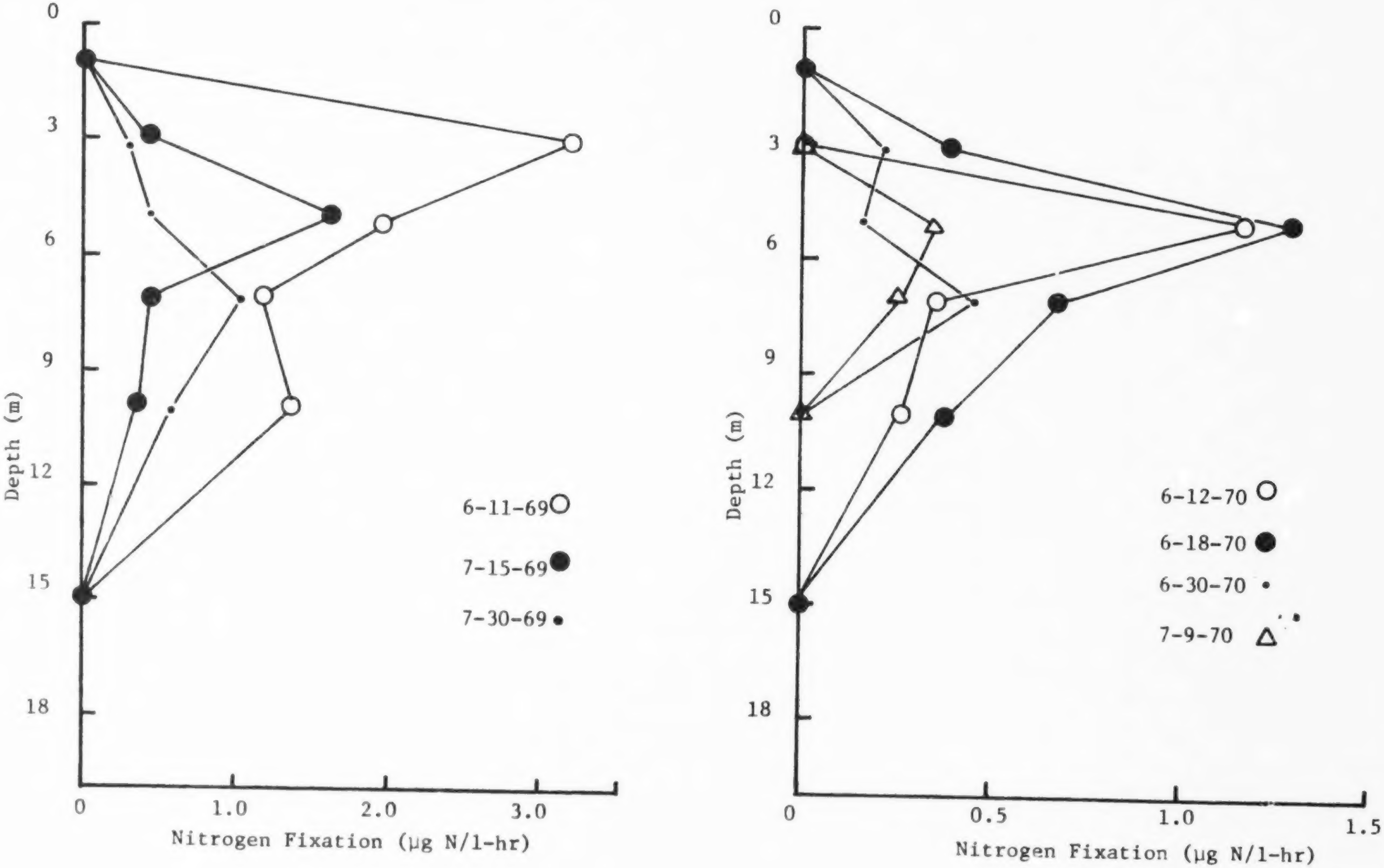


FIGURE 16. DEPTH PROFILES OF NITROGEN FIXATION IN LAKE MIZE DURING 1969 AND 1970.

a shorter period of fixation occurred in 1970 is not completely clear; however the noted nutrient enrichment may have been a factor. Maximum nitrogen fixation rates ranged from 0.08 to 3.26 μg. N/1.-hr.; the upper value is within the range of rates reported for blue-green algae in lakes (Dugdale and Dugdale, 1962; Goering and Neess, 1964; Billaud, 1968).

Nitrogen fixation in Lake Mize is apparently restricted to mid-summer stratification. No fixation was detected in samples assayed in April, September, October, and December 1969 as well as February, April and May 1970. The annual cycle of nitrogen fixation for 1969 and 1970 is illustrated in Figure 17, which shows total hourly nitrogen fixation in the lake over the year. It is apparent that fixation was much greater during 1969 both in duration and rate. Values for total nitrogen fixation per hour were estimated by summing the product of the volumetric fixation rate at each depth times the calculated lake volume for each depth. The annual input of nitrogen to Lake Mize by fixation can be extrapolated from the data in Figure 17. Calculation of yearly rates from a few one-hour incubations is undoubtedly risky, but even an approximate value would be instructive. Assuming that during the period of fixation the reaction occurred 24 hours a day at the rates shown in Figure 16, the total nitrogen contribution in 1969 was 39.2 kg. and in 1970 was 9.6 kg., or 1.14 and 0.28 g./m.^{3} of lake water per year, for 1969 and 1970, respectively. Shannon and Brezonik (1971a) computed a nitrogen budget for Lake Mize (excluding fixation) of 2.05 g./m.^{3}-yr. (see Section V). Thus fixation represents about 56 and 14 percent of the total nitrogen income from other sources in 1969 and 1970, respectively, and at least for this admittedly unusual lake, bacterial nitrogen fixation is a highly significant nutrient source.

Because of the unusual nature of the above results an intensive effort was undertaken to determine the agents of fixation in Lake Mize. Bacteriological samples were collected aseptically at the same depths assayed for nitrogen fixation on several occasions, and samples from those depths where fixation occurred were subjected to enrichment culture for groups of nitrogen-fixing organisms. Three enrichment schemes were utilized covering the spectrum of likely microbial agents: 1) anaerobic or facultative heterotrophic bacteria, 2) photosynthetic bacteria, 3) yeasts and fungi. Details of the enrichment and isolation procedures are given in Appendix C.

Enrichment techniques indicate that at least two nitrogen fixing groups of bacteria, one heterotrophic, the other autotrophic, exist in the depths of Lake Mize. Six isolates of gram positive spore forming rods which grew anaerobically but not aerobically on nitrogen free media were isolated from water samples taken at 3, 5 and 7 meter depths on June 18 and at 5 and 7 meters on July 9. A total of seven transfers of the anaerobic isolates were made, each time to nitrogen free media, and the tests for acetylene reduction were positive at each step. Culture of the six isolates grown in liquid media for 24 hours gave acetylene reduction rates in the range 1.5 to 10 nmole C_2H_2 reduced/mg organism N-hr.

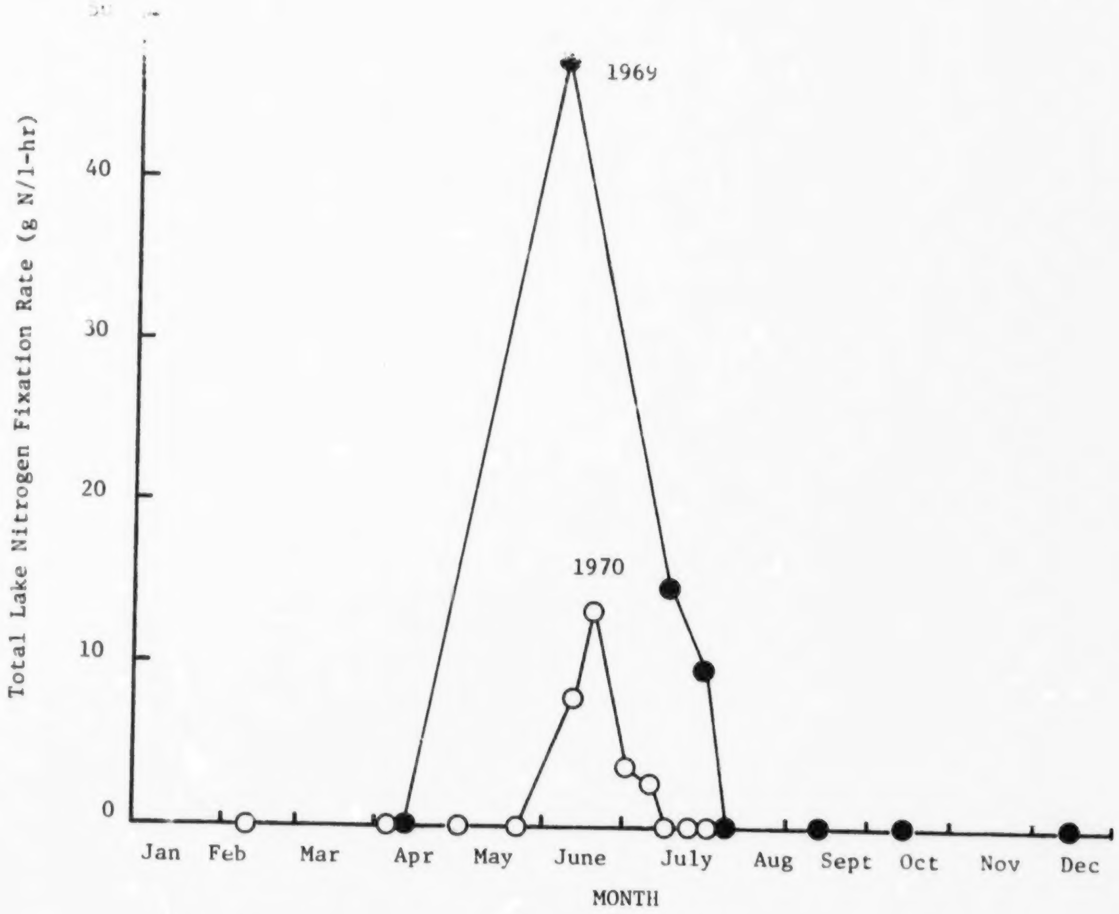


FIGURE 17. HOURLY RATE OF NITROGEN FIXED IN ENTIRE VOLUME OF LAKE MIZE DURING 1969 AND 1970.

Colonies of purple sulfur bacteria began to appear after 4 weeks incubation in water from seven meters depth. Growth began at the glass cellulose interface, which hampered observation of morphological types. Transfers of pigmented colonies were made into liquid media containing only Na_2S and other inorganic salts at pH 8.5 and incubated in the light. Phase contrast microscopy showed two morphological forms, a long motile, spiral shaped organism which we identified as a species of Thiospirillum and a small motile rod, apparently a species of Chromatium. The latter form predominated in mixed cultures and grew rapidly under the culture conditions.

No growth on nitrogen free media occurred within 7 weeks in any of the 65 yeast and fungal isolates picked from the June 18 and July 9 samples. However, growth was observed within 2 weeks for all isolates incubated in the test medium plus nitrogen.

The agents responsible for fixation in Lake Mize are probably heterotrophic bacteria. Fixation occurs only in a region of low or no light and no dissolved oxygen. The fact that fixation is maximum at intermediate depths suggests photosynthetic bacteria may be the agents, and they are in fact present in the subsurface layers of this lake. However, the high rates of acetylene reduction found in Lake Mize samples incubated in the dark would tend to rule out photoautotrophic forms as dominant agents of fixation since photosynthesis apparently provides the source of energy for fixation by these forms. Rhodospirillum rubrum, for example, has been shown to fix nitrogen anaerobically in the light, but intact cells of this bacterium cease fixing immediately when placed in the dark (Pratt and Frenkel 1959). Further, photosynthetic bacteria were isolated from only one of the sampled depths at which fixation occurred (7 meters). The long lag before significant growths of these organisms were noted in enrichment media and the fact that microscopic examination of raw lake water and seston retained on Millipore filters failed to reveal the presence of photosynthetic bacteria imply very low populations in the lake.

On the other hand, heterotrophic growth on a nitrogen-free medium was rapid for samples from the various depths at which fixation occurred. The taxonomic characteristics of the isolates (i.e. gram-positive, spore-forming, obligate anaerobes, capable of utilizing N_2 as their sole N source are those of the genus Clostridium. Whether this is the only heterotrophic bacterium fixing nitrogen in Lake Mize cannot be answered by the present study. Other enrichment procedures may isolate different forms; Arthrobacter has been suggested as another likely agent (J. Sieburth, personal communication). No aerobic or facultative bacteria and no fungi or yeasts were found which could grow on nitrogen deficient media. Cyanophyceans are not likely to be agents of fixation in Lake Mize since algal nitrogen fixation is related to photosynthesis and conditions at the depths of fixation indicate no oxygen production. No blue-green algae were found in samples examined microscopically. The observed stratification of fixation and its annual periodicity may be influenced by such factors as a requirement for low light and narrow tolerance to high or two sulfide concentrations or Eh values, which form gradients in the anoxic zone of this lake.

Sediments are generally considered to be relatively enriched in nutrients and as such are unlikely environments for nitrogen fixation. However, studies supported by this project have detected fixation in both estuarine and lacustrine sediments from subtropical and tropical environments. In addition the report of Howard et al. (1970) on nitrogen fixation in Lake Erie sediments indicates this phenomenon is not unique to warm climates.

Sediments were collected at various locations in the Waccasassa Estuary and in various lakes with a homemade coring device. Cores were returned to the laboratory and sectioned for profile analyses of nitrogen fixation. Since the sediments were anoxic, purging of N_2 was accomplished with helium (see Apendix A for procedured details).

The optimum incubation time for the estuarine sediments was determined to be one hour. The amount of ethylene production was linear for incubation up to that time, but the rate decreased markedly after that (see Section IX). Rates of ethylene production in embayment sediments ranged from 0.00 to 0.54 nM C_2H_4/g. dry wt.-hr. for all the determinations (over 60) made in this study. A distinct layering was found within a typical sediment core (Figure 18). In the flocculent, unconsolidated 1 to 2 cm at the core surface, no C_2H_2 reduction was found at any time. Significant reduction rates were consistently noted in the next 2 to 5 cm of consolidated, grey-black ooze. From 5 to 20 cm the typical core consists of coarse organic material, which overlies the limerock substrate. Acetylene reduction was consistently found in the bottom zones of sediment cores, but rates were low compared with those in the upper portions.

An areal survey of the Waccasassa embayment sediments showed acetylene reduction to be a consistent phenomenon in the upper sediments. Duplicate cores were taken at eight stations in the embayment, and the upper 2 to 5 cm portion of each was blended to obtain a homogenous sample. Acetylene reduction was then run on a portion of the blended sediment. The distribution of ethylene production rates in the estuary sediments is shown in Figure 19. A range of 0.175 to 0.54 nM C_2H_4/g. dry wt.-hr. was found, but most of the values were near the mean rate of 0.33 nM C_2H_4/g. dry wt.-hr. Expressed in terms of the equivalent amount of nitrogen that would have been fixed (using the theoretical ratio of 1.5 moles C_2H_4 produced per mole NH_3 fixed) the results indicate a range of 1.63 to 5.0 ng N/g. dry wt.-hr.

Several experiments were performed to evaluate the environmental conditions for fixation in sediments and to define the agents of fixation. The effect of a nitrogen-free oxygen atmosphere vs. an anoxic (helium) environment was determined in one experiment on five sediment samples. In all but one case, the rate of ethylene product was slightly higher under a helium atmosphere, but the differences were not as great as expected if strict anaerobes were the fixing agents. Probably the organic sediments exerted a sufficient oxygen demand to maintain anoxic microzones in the samples even in an oxygen atmosphere. It was also found that exposure of incubating samples to light decreased the acetylene reduction activity. The mean rate of ethylene production in five replicates incubated in the light was only one third of the mean value

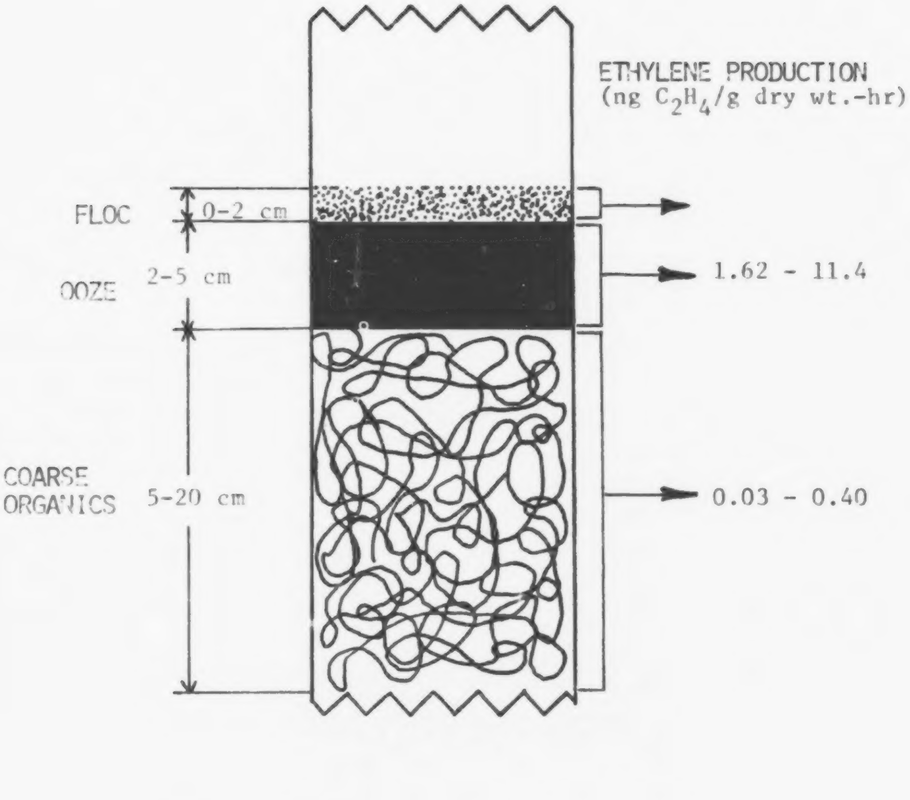
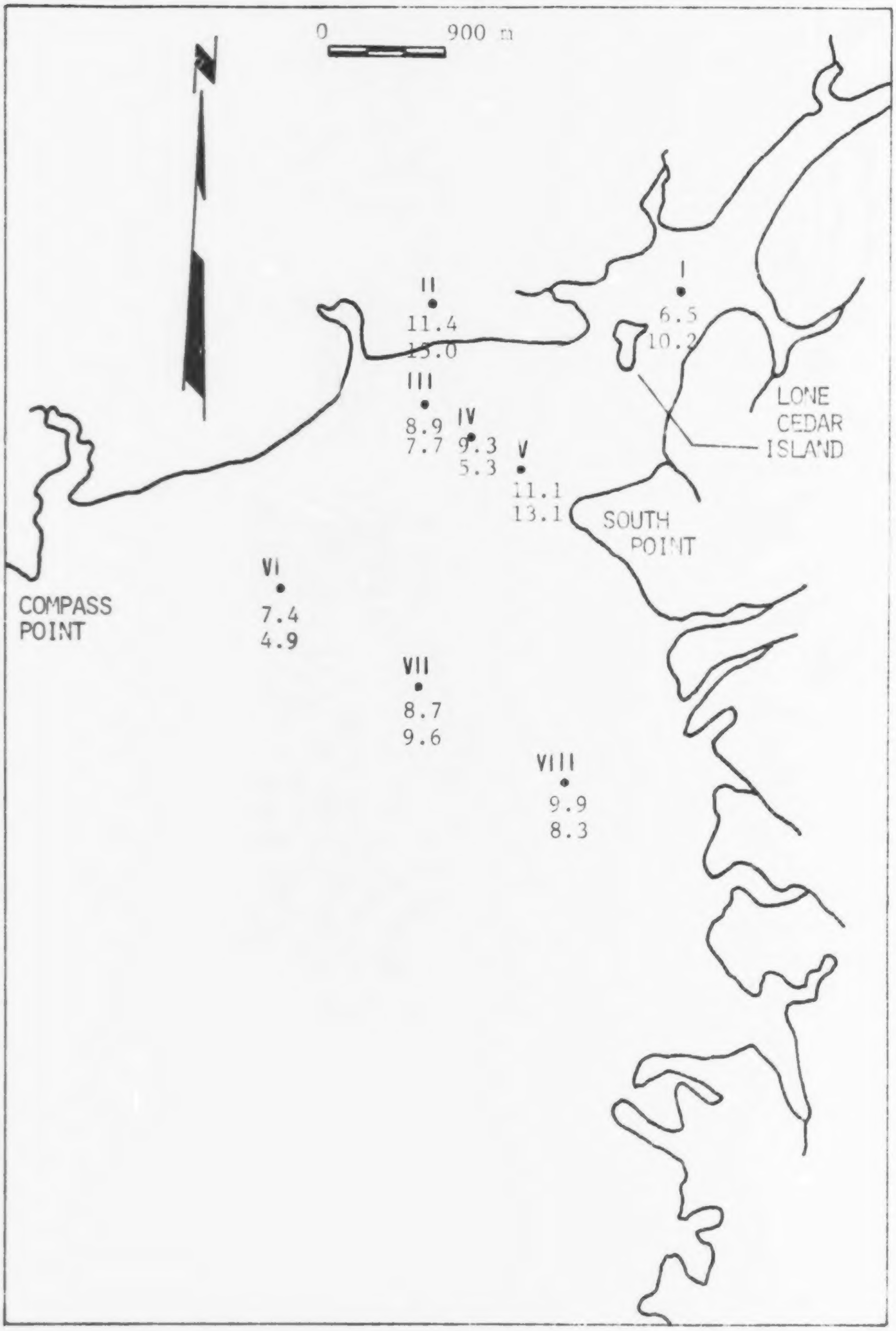

ETHYLENE PRODUCTION
(ng C_2H_4/g dry wt.-hr)
FLOC
0-2 cm
OOZE
2-5 cm
1.62 - 11.4
COARSE ORGANICS
5-20 cm
0.03 - 0.40

FIGURE 18. SECTION OF CORE ILLUSTRATING VARIATION OF ACETYLENE REDUCTION WITH DEPTH IN WACCASASSA ESTUARY SEDIMENTS. RANGE OF ETHYLENE PRODUCED REPRESENTS AT LEAST SIX DETERMINATIONS IN EACH LAYER.


0
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II
11.4
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III
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7.7
IV
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V
11.1
13.1
VI
7.4
4.9
VII
8.7
9.6
VIII
9.9
8.3
LONE
CEDAR
ISLAND
SOUTH
POINT
COMPASS
POINT

FIGURE 19. DISTRIBUTION OF ETHYLENE PRODUCTION RATES WITHIN WACCASASSA ESTUARY SEDIMENTS (in ng C_2H_4/g dry wt.-hr). DUPLICATE CORES AT EACH OF EIGHT STATIONS. STATION II WAS IN MUD FLAT.

for replicates of the same sediment incubated in the dark. The reasons for this cannot be fully stated; however, it is well known that many non-photosynthetic bacteria have pigments and that these organisms can be inhibited by visible radiation.

Since the agents of ethylene production (hence nitrogen fixation) in the sediments are presumably anaerobic or facultative bacteria, addition of easily assimilable carbonaceous substrate to sediment samples should enhance their activity. Several experiments have given somewhat conflicting results in this regard. In an early experiment acetate gave apparent stimulation of acetylene reduction at concentrations of 0.02 and 0.2 M but glucose gave no response. However no acetate stimulation was found in three later experiments in the range 10^{-4} to 10^{-1} M, and in fact 10^{-1} M acetate and butyrate actually inhibited fixation by about 30 percent. No evidence for glucose stimulation or inhibition was found in any of the four enrichment experiments in the concentration range 10^{-4} - 10^{-1} M, but sucrose gave a definite stimulation (50-100 percent increases over controls) in each of the three experiments in which it was added (Table 19). These results are similar to responses of lacustrine sediments to organic additions (described later in this section).

Several experiments were performed to evaluate the impact of inhibitors on acetylene reduction. In 30-40 ml. sediment slurries fixation was inhibited by 1 ml. of fifty percent trichloroacetic acid or 3 ml. of saturated mercuric chloride solution. If acetylene reduction is related to nitrogen fixing activity, it would be expected that molecular nitrogen would inhibit the rate of acetylene reduction. Since acetylene is a competitive inhibitor of N_2 (Schöllhorn and Burris, 1967), the converse should be true, i.e. added molecular nitrogen should reduct the rate of acetylene reduction according to the competitive enzyme inhibition pattern (see Cleland (1963) for a complete discussion of enzyme kinetics). Thus a reciprocal (Lineweaver-Burk) plot of reaction velocity (ethylene production rate) versus substrate (acetylene) concentration should be linear, and added inhibitor (molecular nitrogen) should give the classical competitive inhibition plot (see Section IX). To verify this for Waccasassa sediments, two sets of samples were set up; one series was exposed to various concentrations of acetylene in a helium (nitrogen-free atmosphere, and in another series using the same sediment, samples were exposed to various acetylene concentrations in a 70% N_2 - 30% CO_2 atmosphere. A Lineweaver-Burk (reciprocal) plot of the ethlene production rate vs. acetylene concentration (Figure 20) shows the classical competitive inhibition pattern. The maximum velocity (V_{max}) as obtained from the y-intercept of the Lineweaver-Burk plot is approximately the same for both curves implying that high concentrations of acetylene negate the inhibitory effect of N_2. Competitive inhibition patterns are given by enzyme inhibitors that react at the same active site on the enzyme as the substrate.

The organisms most likely to be responsible for the above phenomena would seem to be anaerobic bacteria such as Clostridium sp. An enrichment and isolation procedure was conducted (see Appendix C for details on methodology) to determine whether such organisms could be

Table 19. Effect of Organic Substrate Addition on Ethylene Production by Estuarine Sediment

	Relative Response[1] Concentration Added (Molar)			
Substrate	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Acetate	-	0	0	0
Butyrate	-	0	0	0
Glucose	0	0	0	0
Sucrose	+	+	0	0

[1] Response relative to controls with no added substrate: inhibition (-); no change (0); stimulation (+) of acetylene reduction to ethylene.

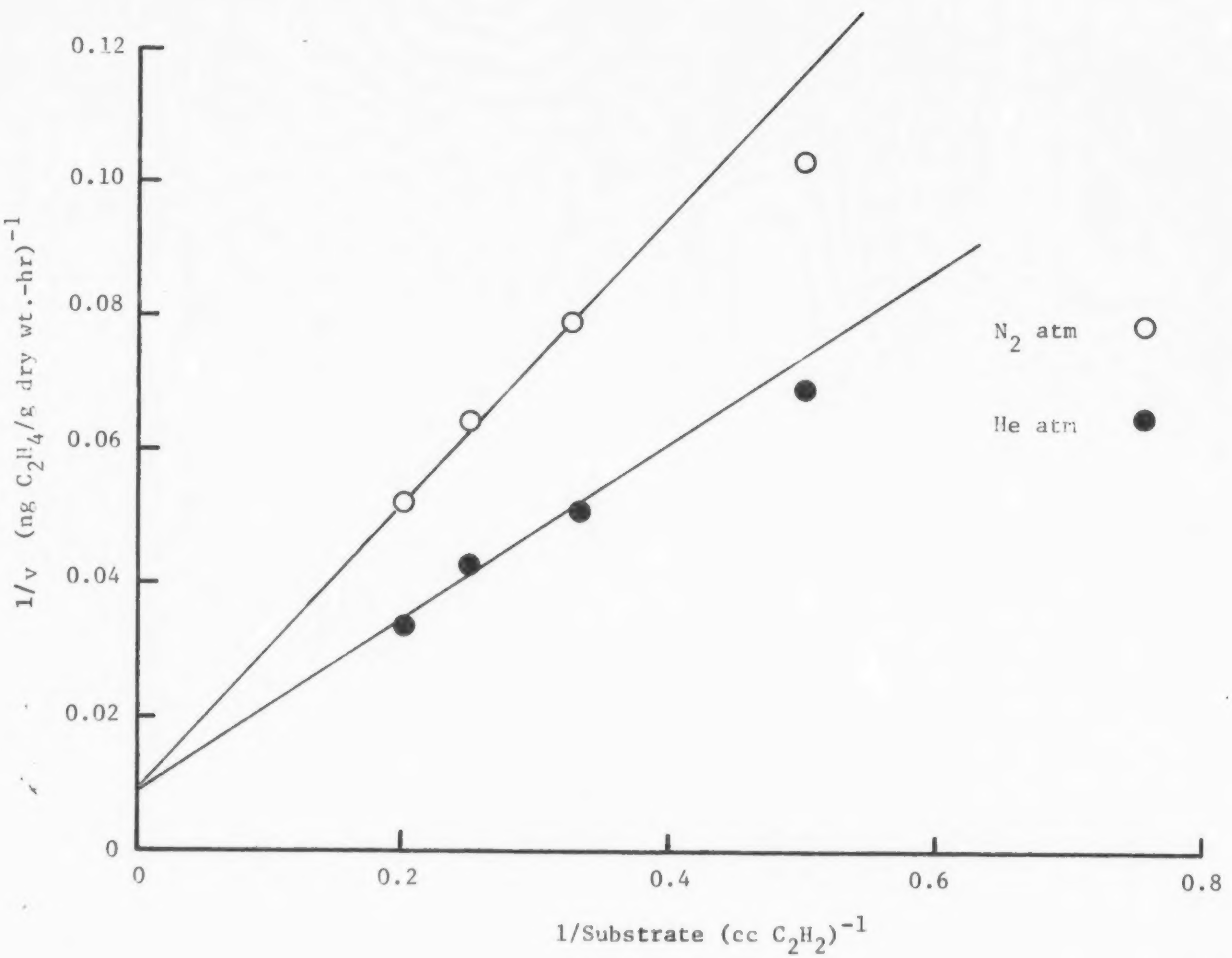

FIGURE 20. LINEWEAVER-BURK PLOT OF ETHYLENE PRODUCTION VS ACETYLENE CONCENTRATION INDICATES COMPETITIVE INHIBITION BY N_2 IN WACCASASSA ESTUARY SEDIMENT.

detected in Waccasassa sediments. Three isolates were obtained which could grow in a nitrogen-free medium and reduce acetylene to ethylene (hence presumably fix molecular nitrogen). The characteristics of the final isolates were highly indicative of a Clostridium (or clostridium-like) culture; i.e. the isolates were gram-positive, strict anaerobic, large rods, which ferment sugars (and fix molecular nitrogen). Further physiological tests would of course be necessary to establish specific taxonomy of the cultures, especially down to the species level. However the taxonomy of marine and estuarine anaerobes is not highly developed, and to place the cultures in a particular genus would be questionable at this time. Nonetheless, this work has demonstrated that "clostridia-like" nitrogen-fixing bacteria are present in Waccasassa Estuary sediments. The results do not exclude the possibility of other nitrogen-fixing forms being present in the sediment since the isolation method used was somewhat selective for clostridia-like bacteria.

Sufficient evidence has been obtained to conclude with reasonable assurance that the acetylene reduction activity is directly related to nitrogen fixing organisms in the sediments. All lines of evidence point to ethylene production as a biological phenomenon; both trichloroacetic acid and mercuric chloride completely inhibit acetylene reduction. Organic substrate (in some cases) affects the rate of ethylene production. The fact that N_2 acts as a competitive inhibitor of acetylene reduction strongly suggests that ethylene is produced by nitrogenase. Nitrogen-free media produced growths of gram positive spore-forming rods from sediments under an atmosphere of pure nitrogen, and a pure culture similar to Clostridium sp. was isolated from the sediment and was shown capable of nitrogen fixation by acetylene reduction

A rough estimate of the total amount of nitrogen fixed in the 2-5 cm layer of sediments in the Waccasassa can be obtained from the data presented in Figure 19. Assuming that the mean value of 3.1 ng N fixed/g sediment-hr. is a reasonable estimate of the nitrogen fixation rate in this stratum of sediment throughout the estuary, the amount of nitrogen fixed on an annual basis is 37 μg N cm^2-yr. or for the entire 7 km^2 estuary, 2.6×10^3 kg N/yr. These values are based on an average sediment dry weight of 0.455 g/cm^3 in the 2 to 5 cm zone. These extrapolated values are obviously rather crude, but they serve to illustrate the point that the seemingly low rates found in the sediments in fact represent significant amounts of nitrogen fixed on an annual basis.

Because sediments are normally thought to be enriched in nutrients, we were somewhat surprised at our initial results indicating nitrogen fixation in Waccasassa estuary sediments. Free ammonia was determined on sediments from 14 widely scattered stations in the estuary (Brooks, 1969); a range of 0.01 to 0.37 and mean of 0.06 mg NH_3-N/g sediment was found. Not all this ammonia would necessarily be available to microorganisms; much or perhaps all of it could be loosely sorbed to solid material in the sediment. Some evidence was found to support this hypothesis (see Section VIII); Waccasassa Estuary sediment was shown to contain 3 to 5 percent clay mineral and to sorb ammonia rapidly from solution.

The first indication that lacustrine sediments supports nitrogen fixing activities was uncovered in 1968 when a short core from Bivin's Arm yielded positive rates which decreased rapidly with depth (Table 20). Sediment cores were obtained from 16 lakes in north central Florida in September, 1969, and from these and 9 additional lakes in June and July of 1970 to determine whether lacustrine sediment fixation is a more widespread phenomenon. These lakes were all sampled periodically in conjunction with other studies (Shannon and Brezonik, 1971c; Brezonik, 1971c), and their water and sediment characteristics are thus known. A list of the 25 lakes and their trophic states is presented in Table 21 along with pertinent sediment characteristics. Three general types of sediment occur in the lakes: A) brown flocculent or unconsolidated material often with undecomposed plant remains, B) black, jelly-like sediment, and C) sandy sediment.

Short cores (30-50 cm) from the lakes either blended in their entirety and an aliquot taken for measurement of nitrogen fixation or segmented into several zones which were then tested for fixation individually. Another aliquot of sediment was dried and weighed, and results were reported as rate of fixation per gram dry weight of sediment. Seven of the twenty-five lake sediments showed apparent nitrogen fixation in one or both surveys (Table 22). Four of the seven sediments were peat-like (Type A), and except for Lake Apopka, these also gave the highest rates of ethylen production. Peat sediments might be expected to have a lower available nitrogen content than muck sediments. Sediment from lake #20 appeared tobbe a mixture of sand and sludge. This shallow lake (maximum depth is 3.5 m) receives some domestic waste effluent and is at times anoxic below 1 meter. Sediments from Lakes Apopka, Bivin's Arm, Kanapaha and Orange emitted a musty or moldy odor when blended, while the other sediments were either odorless or gave off a hydrogen sulfide odor. Lake Alice was the only sediment with a pronounced H_2S odor that showed significant fixation. This lake receives a large proportion of its inflow as treated sewage effluent. Five cores taken from this lake in 1969 showed fixation, but no fixation was found in a core taken during the 1970 survey.

In order to determine the layers of sediment most active in nitrogen fixation, profiles of acetylene reduction rates were measured on sediment cores from Orange Lake in the 1969 survey and on all lakes sampled in the 1970 survey. The loose, unconsolidated nature of most of the sediments exhibiting nitrogen fixation precluded detailed segmenting of the cores, and only the core from unnamed lake 20 was easily divisible into segments. Table 23 presents the 1969 profiles for Orange Lake and profiles from the 4 lakes exhibiting fixation in the 1970 survey. Highest rates were found in the upper layers; this trend was most marked when the rates are considered per gram dry weight of sediment rather than per milligram of sediment nitrogen. Sediment from lake #20, while not as active as the other three sediments when compared on a sediment dry weight basis, showed comparable activity on per sediment nitrogen basis.

Sediment samples from lakes in the Peten region of Guatemala surveyed in conjunction with another project during the summers of 1969 and 1970 also showed nitrogen fixation by the acetylene reduction method. Locations and descriptions of these lakes are given by Fox et. al. (1970).

Table 20. Acetylene Reduction in
Bivin's Arm Sediment Collected in August, 1968

Sample	Treatment[1]	Ethylene Production nM C_2H_4/g. dry wt-hr.
Ekman dredge - Surface to 3" mixture	Control (TCA) He[2]	0.043
	He	2.09
	O_2,Ar	0.54
	500 mg NH_3-N/l added, He	0.27
Core		
Surface	O_2,Ar	0.31
Surface	He	2.15
3" deep	He	0.25
5" deep	He	0.21
7" deep	He	0.10
9" deep	He	0.09

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[1] Control treated with 1 ml 50% trichloroacetic acid before adding acetylene. Samples purged either with helium (He) or with gas mixture (20% O_2, 0.03% CO_2, balance Ar) to remove dissolved N_2.

[2] Equal to "blank" reading, i.e. background ethylene present in acetylene.

Table 21. Lakes Surveyed for Sediment Nitrogen Fixation[1],[2]

Hypereutrophic

Apopka (B)
Unnamed 20 (D)
Bivin's Arm (A)
Griffin (B)
Alice (A)
Eustis (B)
Kanapaha (A)

Eutrophic

Hawthorne (A)
Clear (B)
Wauberg (A)
Newnan's (A)

Mesotrophic

Cooter Pond (B)
Lochloosa (B)
Calf Pond (A)
Orange (A)

Oligotrophic

Watermelon Pond (B)
Unnamed 10 (A)
Jeggord (B)
Moss Lee (B)
Altho (B)

Ultraoligotrophic

Anderson-Cue (A)
Gallilee (B)
Cowpen (B)
Sand Hill (C)
Swan (C)

[1] Classification into trophic class after Shannon and Brezonik (1971b).

[2] Sediment types found:

A-Brown flocculant unconsolidated material with or without undecomposed plant remains.
B-Black Jelly like sediment.
C-Sandy bottom.
D-This sediment was a mixture of sand and brown, foul-smelling sludge.

Table 22. Nitrogen Fixation Rates in Florida Lake Sediments

Lake	Sediment Characteristics	Nitrogen Fixation Rate[1] 1969 ng.N/g-hr.	1970[2] ng.N/g-hr.	μg.N/g N-hr.
Bivin's Arm	A	28	22	2.0
Kanapaha	A	36	59	1.4
Orange	A	9.5	28	1.3
Moss Lee	B	1.8	0	0
Apopka[3]	B	17	-	-
Alice	A	1.2[4]	0	0
Unnamed 20	D	0	16	23

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[1] Calculated assumed a C_2H_4/NH_3 molar production ratio of 3/2.

[2] Maximum rate found in profile (Table 23).

[3] One dredge sample Nov. 3, 1969.

[4] Average of 5 cores.

Table 23. Stratification of Nitrogen Fixation in Florida Lake Sediments

Stratum (cm)		Nitrogen Fixation Rate	
		ng N/g - hr.	µg N/g N-hr.
	Orange Lake 1969		
0-37	Shallow Water Core	1.09	----
37-50		0.33	----
0-36	Deep Water Core	0.98	----
36-50		0.29	----
	1970		
0-33		28	1.3
33-42		1.1	0.78
	Lake Kanapaha 1970		
0-5		59	1.4
5-15		10	1.4
15-25		3	0.37
25-38		0	0
	Lake Bivin's Arm 1970		
0-2		14	1.4
2-5		22	2.0
5-10		18	1.9
10-15		5	0.8
20-25		0	0
	Lake Unnamed 20 1970		
0-2		3	0.51
2-4		12	2.1
4-6		16	2.3
6-8		3	0.71
8-10		4	0.25
10-12		6	0.24
12-15		0	0
15-20		0	0
20-25		0	0

In 1969, Laguna Petenxil sediment fixed at a rate of 3.1 μg. N/g. sediment N-hr., while green flocculant surface sediment from Laguna Echixil fixed at a rate of 5 μg. N/g. sediment-hr. Sediments from two Petén lakes (Eckixil and Sal Petén) were sampled in detailed profiles in 1970, and high rates of fixation were found in both lakes (up to 2.4 μg. N/g. sed. N-hr.). Sal Petén sediment exhibited three distinct bands of fixation. The near surface sediments of these lakes are unusual in being composed of flocculent, pigmented organic particles. The green to pink color of the sediment apparently derives from undecomposed algae and perhaps partially from photosynthetic bacteria.

Because the acetylene reduction technique is an indirect assay for nitrogen fixation and because fixation had never before been measured in a sedimentary environment, it was felt essential to demonstrate that the measured ethylene production indeed reflected nitrogen fixing activities. This was accomplished by a variety of experiments. First, biological poisons were shown to inhibit acetylene reduction. Addition of 1 ml of 50% trichloroacetic acid or 3 ml of saturated mercuric chloride to sediment samples (25 ml slurries) immediately and completely stopped ethylene production. Second, if acetylene reduction is related to nitrogen fixation, addition of N_2 should inhibit the rate of acetylene reduction as was shown for Waccasassa Estuary sediments and for nitrogen fixing algae (see Section IX). A similar experiment was performed with sediment from Lake Kanapaha, a shallow, highly eutrophic lake near Gainesville, Florida. Varying amounts of acetylene were added to serum bottles containing 25 ml of sediment slurry which had either been purged with an O_2-Ar mixture to eliminate N_2 or left unpurged, and the samples were incubated for 1.5 hours at 22°C in the dark. A Lineweaver-Burk (reciprocal) plot of the resulting rates vs. substrate (acetylene) concentrations (Figure 21) fit a pattern of competitive inhibition of ethylene production by N_2. This corroborates the experiment on Waccasassa Estuary sediment discussed previously and is in agreement with Schöllhorn and Burris' (1967) finding that acetylene is a competitive inhibitor of nitrogen fixation.

Heterotrophic nitrogen fixation is known to depend on the availability of organic carbon. Table 24 shows the results of an experiment designed to show the effect of various organic substrates on acetylene reduction in lake sediment. Twenty-five ml. samples of sediment slurry were purged and injected with acetylene in the usual manner except that prior to injection of acetylene sufficient carbon source was added to give the concentrations shown. Sucrose definitely stimulated acetylene reduction while the 0.10 M concentrations of sodium acetate and butyrate caused inhibition. The above experiments strongly support a biological mediation of ethylene production from acetylene which is directly related to nitrogen fixing activities. Fixation in both estuarine and lacustrine sediments is most likely mediated by heterotrophic bacteria, and sucrose additions stimulated fixation in both sediments. Photosynthetic bacteria are obviously unlikely agents for the observed activities since fixation extends into fairly deep layers of sediment and blue-green algae are rare or absent in the Waccasassa Estuary and in some of the lakes whose sediments show fixation. Blue-green algae cannot be completely ruled out in all cases, for Hoare *et al*. (1971) have demonstrated that *Nostoc*

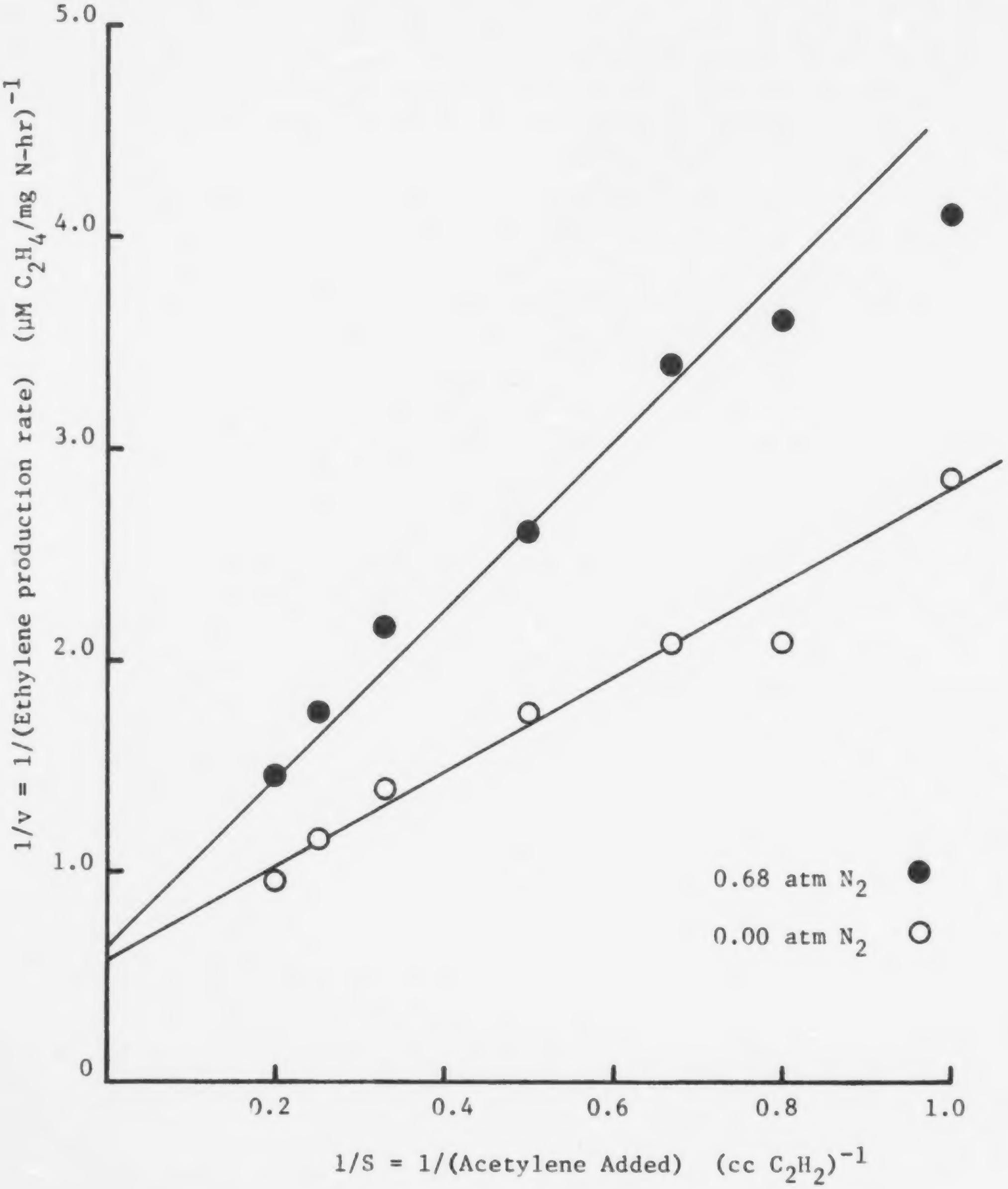

FIGURE 21. COMPETITIVE INHIBITION OF ETHYLENE PRODUCTION (BY NITROGENASE) CAUSE BY N_2 IN LAKE KANAPAHA SEDIMENT.

and other species can metabolize heterotrophically even under anoxic conditions. However, bacteria are at least as likely an explanation for fixation under these circumstances.

While nitrogen fixation does not occur universally in lake sediments, its occurrence at least sometimes in 7 of the 25 Florida lake sediments suggests that the phenomenon is more than just an environmental curiosity. The occurrence of fixation in the three Guatemala sediments tested in Lake Erie and Waccasassa Estuary sediments indicates a fairly widespread distribution of low nitrogen fixation rates in the sediments underlying natural waters. The range of fixation rates measured in Florida sediments was 0.33 - 59 ng. N/g. sediment-hr. in surface layers and 0.02 - 1.1 ng. N/g. sediment-hr. in the bottom strata of 30-50 cm. cores. Comparable rates were found in Guatemalan lake sediments and in sediments from the Waccasassa Estuary. The rates for Lake Erie sediments (Howard et al., 1970) are also in the same range.

It would be of great interest to determine why fixation occurs in some sediments but not in others. Examination of sediment characteristics of the Florida lakes shows no obvious correlation between the occurrence of sediment fixation and sediment ammonia, total organic nitrogen, phosphorus, or percent volatile solids, carbon or nitrogen. Fixation activities should be inversely related to ammonia concentration. On the other hand, even though sediments may be apparently nitrogen-rich, much of the ammonia may be sorbed onto clays and other particles and may unavailable to microorganisms (see Section VIII).

Increased activity in the upper layers of sediment probably reflects higher concentrations of oxidizable substrate in these layers. Depth profiles of fixation were not nearly so narrow in the lake sediment cores as those found in Waccasassa Estuary sediments, where fixation was confined largely to the 2-5 cm. stratum. The broader depth distribution is undoubtedly related to wind-induced mixing of relatively unconsolidated sediments in the shallow lakes. This hypothesis is supported by the results for lake #20, which had a sharper stratification and which also has a fairly compact sediment. Stratification of fixation is much more pronounced when the rates are expressed per gram dry weight sediment than when expressed per unit of organic nitrogen in the sediment.

Nitrogen fixed in the sediments will be only partially released to the overlying water, depending on the degree of mixing effected by wind action. In the loose unconsolidated sediments such as found in Lakes Kanapaha, Orange and Bivin's Arm, this may be considerable; in compacted sediments release to the overlying water may be controlled by much slower diffusion processes. Extrapolation of the sediment fixation rates in Tables 22 and 23 to annual amounts indicates that the process may contribute substantial quantities of nitrogen to the lake basin as a whole and may in this sense be geochemically significant.

SECTION VIII

OTHER IN SITU NITROGEN SOURCES AND SINKS

Although sediments are generally described as nutrient traps, this is only true as a net reaction. Transfer of nutrients between water and sediment is at least partially reversible and to an extent cyclic rather than one-way. Over geological time sediments build-up acts as a net sink, but on a seasonal or shorter time basis the possibility of sediments acting as nutrient sources must also be considered. Nitrogen is lost to sediments by deposition of particulate matter (detritus, silt) and by sorption of ammonia onto clays in or being deposited onto the sediments. Nitrogen can be released from sediments by activities of burrowing animals, decomposition of organic nitrogen to ammonia and its diffusion into the overlying water, and desorption of ammonia from clays and other sorbents in the sediment.

Nitrogen exchange at the sediment-water interface has received scant attention compared to phosphate exchange. Several classic studies have discussed the role of oxygen and redox potential in controlling diffusion of ammonia from sediments. Mortimer (1941, 1942) reported that little ammonia is released from sediments as long as an oxidized microzone exists but that large quantities of this (and other nutrients) are liberated when this surface layer is reduced. He felt that this general increase could be explained by sorption of ammonia onto a complex organic floc containing iron (III). The apparently low rate of nitrogen release from oxidized sediments could also be partially explained by nitrification at the sediment surface causing nitrate rather than ammonia release, and by the fact that ammonia is unlikely to accumulate in oxygenated water as it will either be oxidized or assimilated. The role of the oxidized microzone in influencing sediment-water nutrient cycling would be a fruitful area for future research.

For purposes of nutrient exchange, lake sediments can be divided into at least two main types based on the presence or absence of a defined water-sediment interface. In the former case nutrient exchange may be limited by rates of diffusion, the presence of an oxidized surface layer, and the activities of burrowing animals (Gahler, 1969). Many shallow, eutrophic Florida lakes have no defined sediment-water interface. A flocculant suspension covers the bottom in these lakes with gradual compaction from thin "soup" to consolidated sediment occurring over a depth of perhaps several feet. In this case considerable exchange may be effected by wind generated currents and turbulence, causing the sediment suspension to mix with overlying water. Rates of anaerobic decomposition of organic sediment are probably a primary factor limiting nutrient exchange in such sediments.

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Thus, sediment-water nitrogen interactions involve both biological and chemical processes, and rates of exchange may be further controlled by physical (e.g. diffusion) phenomenon. Qualitative aspects of biological decomposition in sediments are fairly well understood (e.g. Foree et al., 1970; Lee, 1970b; Rittenberg et al., 1955) and need no further elaboration here. The importance of burrowing animals (e.g. worms, larvae, crustaceans) in aerating sediments and in physically transporting nutrients from the sediment and excreting them into the overlying water was suggested (Brooks, 1969) in work supported by this project. When surface sediment from the Waccasassa Estuary (Florida) was covered with estuary water in laboratory aquaria, a defined sediment-water interface and oxidized surface layer quickly arose, and within several days extensive burrowing and tube building by amphipods and annelids were evident through the aquarium glass wall. However quantitative data on regeneration rates induced by this mechanism are lacking.

Ammonia may be sorbed onto clays and organic colloids in sediments, in which case an equilibrium between aqueous ammonia and ammonia in sediments would be set up. Principles of soil chemistry and cation adsorption processes in soils should be applicable to the study of this phenomenon in sediments (Toetz, 1970; Toth and Ott, 1970). Carritt and Goodgal (1954) listed clays, gels of ferric hydroxide and silicic acid, humus colloids, polymorphic inorganic and organic complexes and surfaces of living and dead organisms as possible adsorbents active in sediments. If sorption plays an important role in ammonia equilibrium between sediment and water, it should be possible to describe the equilibrium between ammonia adsorbed and ammonia concentration in the interstitial water by one of the well-known sorption isotherms such as the Langmuir or Freundlich. Since other cations compete with ammonia for sorbent binding sites, the extent of ammonia adsorption should be affected by changes in concentrations of other ions in the bulk solution. On the other hand a considerable amount of "trapping" may occur such that the sorbed ions are not freely in contact with bulk or institial water but have migrated by intraparticle diffusion. Such a situation would render the sorbed ammonia relatively immune to changes in composition of the bulk solution, in effect making ammonia sorption irreversible.

A problem in defining the role of sorption in sediment-nutrient interactions is to separate the effects of biological activities. Use of poisoned or irradiated sediments is possible, but there is always a danger in changing the chemical nature of sediment by such treatment. The importance of chemical processes can be inferred if rapid uptake or release occurs during short (several hour) incubations since biological decomposition and assimilation are inherently slow processes. A further problem also arises in relating the results of laboratory shaker-flask experiments on sorption and leaching to in situ conditions.

The high concentrations of ammonia which typically arise in anoxic lake hypolimnia during summer stratification have long been known (Domogalla et al., 1926; Hutchinson, 1957). Release of ammonia from sediments has been a widely accepted mechanism for these increases, but the decomposition of sinking organisms and detritus offers an alternate explanation for at least some of the increase. Attempts to determine the relative importance of these two mechanisms have not yet been successful. Rates of detrital deposition in sediment traps and changes in the elemental composition of detritus as it sinks have been reported (Kleerekoper, 1953), and sediment leaching experiments involving laboratory incubations have been described by Mortimer (1941, 1942), Sawyer, et al. (1945) and others. But the quantitative significance of sediments as an ammonia source remains elusive. Recently Fitzgerald (1970) described experiments in which algae grown in contact with sediment from Lake Mendota, Wisconsin, maintained a phosphorus deficiency in spite of the high phosphate level of the sediments. The results qualitatively suggest that phosphate incorporation into sediments is largely irreversible. Whether this also applies to nitrogen in sediments and if so, under what conditions, remains unknown.

In summary the processes of nitrogen cycling and interchange in sediments are complicated and poorly understood. The mechanisms whereby nitrogen is exchanged between water and sediments are probably known, and in some cases qualitative rankings can be given to their importance. However almost no information is available to establish the in situ rates and controlling factors for these processes.

The chemistry of nitrogen in Florida sediments has been studied in two phases. The chemical characteristics of sediments from the 55 lakes described in previous sections have been evaluated to provide a basis for later studies on the dynamics of nutrient exchange. In conjunction with a separately funded project the sediments were analyzed for total organic nitrogen, free ammonia, ortho and total phosphate, iron, manganese, volatile solids, C, H, and N contents by elemental analysis, chlorophyll and carotenoid degradation products, benthos, and gross physical description. Of primary concern here are the first two analyses, but the other parameters are important in describing the sediments and may later be useful in relating exchange rates and capacities to sediment type.

Results for surface sediments collected by Ekman dredge from the lakes are presented in Table 25. For comparative purposes the type of sediment and organic content are listed along with nitrogen content. Methods used in the analyses are described in Appendix A. Highest concentrations of sediment ammonia were generally associated with enriched or polluted lakes (e.g. Hawthorne, Dora, Griffin) or with undisturbed lakes having a high sediment organic content. Ammonia concentrations ranged from less than 10 ppm to over 1000 ppm on a dry weight basis. Total nitrogen values ranged from 0 to 4.0 percent on a dry weight basis, but low levels (less than one percent) were generally associated with low carbon and low volatile solid samples. For example, no nitrogen was detected in the Swan Lake (Putnam County) sample, but this sediment was composed largely of sand and had only 1.2 percent carbon and 2.3 percent volatile solids. This lake is also very clear and can be considered ultra-oligotrophic. Highest values of total nitrogen were associated with high volatile solids and carbon and usually with eutrophic or polluted conditions. Lake Hawthorne had the highest sediment nitrogen; this eutrophic lake receives sewage effluent from the town of

Table 25. Sediment Characteristics of North Central Florida Lakes

Lake [1]	Sediment Type[2]	Volatile Solids%	Ammonia-N[3] µg/g	Carbon[3] %	Nitrogen[3] %	C/N wt./wt.
Sante Fe	DBr,Bl-N-Si	18.7	30	11.5	0.7	16.6
Little Santa Fe	DBr,Bl-N-Si	58.8	30	45.2	2.4	18.7
Hickory Pond	DBr-O-S,Si	6.8	10	4.6	0.3	18.4
Altho	DBr-O-S,Si	61.5	40	36.6	1.8	20.6
Cooter	DBr-O-S,Si,Or	51.5	70	26.6	2.1	12.7
Elizabeth	DBr-T-S,Or	13.2	10	8.1	0.4	19.6
Clearwater	Br-O-S,Or	8.1	10	6.1	0.4	15.3
Hawthorne	LBr-N-S,Or	82.4	1010	44.9	4.0	11.1
Little Orange	DBr-T-S,Or	15.0	80	8.2	0.6	14.8
Unnamed (#10)	LBr-N-S,Or	88.3	180	49.1	3.5	14.1
Moss Lee	DBr-T-S	87.3	180	49.6	3.5	14.3
Jeggord	DBr-T-S	62.2	20	42.3	1.4	29.6
Still Pond	LBr-O-S,D	24.7	140	1.6	0.1	12.4
Lochloosa	Br-O-S,Or	15.3	10	8.7	0.9	9.8
Orange	Br-O-S,Or,P	47.9	50	26.0	2.4	11.0
Palatka	Br-N-D	95.1	520	48.2	3.1	15.5
Newnan's	Br-O-P	52.8	320	28.1	2.4	11.9
Calf	Br-N-P,D	84.3	10	50.1	3.5	14.2
Unnamed (#20)	G-O-S	25.7	310	12.8	0.9	14.4
Meta	G-O-S,D	29.6	30	18.3	1.3	14.4
Alice	Br-O-P,D	43.0	40	14.8	1.2	12.7
Bivin's Arm	Br-O-P,D	65.7	570	35.5	3.2	11.2
Clear	Br-O-S	4.9	10	2.3	0.2	11.7
Unnamed (#25)	Br-O-S,D	2.1	10	1.2	0.1	13.7
Beville's Pond	Br-O-P,D	84.4	270	46.2	3.9	11.9
Unnamed (#27)	Br-O-P,D	41.7	50	23.6	1.9	12.7
Kanapaha	Br-O-P,D	76.5	240	40.3	3.6	11.2
Watermelon	Bl-N-M,S	64.6	60	43.4	2.9	14.8
Long Pond	Bl-O-M,D	49.6	60	27.3	2.1	12.8
Burnt	DBr-O-S,M,D	34.5	60	18.6	1.3	14.2
Wauburg	Bl-O-S,Or	39.2	460	23.6	1.7	13.6
Tuscawilla	G,Br-O-S,D	52.7	80	27.3	2.1	13.1
Apopka	DBr-O-S,M	52.2	250	29.7	2.4	12.5
Dora	DBr-O-S,M	59.7	480	27.4	2.2	12.5
Harris	DBr-O-S,M	32.5	250	16.9	1.6	10.6
Eustis	DBr-O-S,M	56.4	150	26.9	2.3	11.7
Griffin	DBr-O-S,M	63.4	820	33.7	2.7	12.3
Weir	G,Br-O-S,Si	54.7	150	31.8	2.4	13.3
Kingsley	Bl,Br-O-S,Si	34.4	30	18.3	1.1	17.4
Sumter Lowry	Bl-O-S,Si,D	24.4	10	14.2	0.7	20.0
Magnolia	Bl-O-S,Si	32.2	30	14.9	0.7	22.6
Brooklyn	Bl-O-S,Si	7.4	10	3.3	0.2	15.0
Geneva	Bl-O-S	16.6	< 10	8.9	0.6	15.8

Table 25. (Contd.)

Lake	Sediment Type[2]	Volatile Solids%	Ammonia-N[3] μg/g	Carbon[3] %	Nitrogen[3] %	C/N wt./wt.
Swan	Bl-O-S	2.3	<10	1.2	0.0	--
Wall	Br-O-S,P	66.8	30	41.5	2.6	16.3
Santa Rosa	LBr-N-S	14.9	410	0.4	0.02	21.0
Adaho	DBr-O-S,Si	58.9	30	35.9	1.8	19.5
McCloud	DBr-O-S,Si	67.4	510	38.4	2.4	16.5
Anderson-Cue	DBr-O-S,Si,D	85.3	80	50.2	2.7	18.5
Suggs	DBr-O-S,Si	40.5	50	6.4	0.4	17.3
Long	DBr-N-S	31.6	150	0.7	0.03	24.3
Cowpen	Bl-O-S,Si	31.4	10	18.9	1.2	16.0
Gallilee	Bl-O-S,Si	49.2	20	29.4	1.8	16.5

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[1] Lakes Santa Fe to Tuscawilla are in Alachua County; Lakes Apopka to Weir are in the Oklawaha River Basin; remaining lakes are in the Trail Ridge (sandhill) region of the central highlands. See Brezonik et al. (1969) for lake locations.

[2] Sediments typed according to color, odor, and texture: Br=brown, DB=dark brown, LBr=light brown, Bl=black, G=gray, O=H_2S odor, N=No odor, S=sand, Si=silt, Or=fine organic matter, P=peat, D=debris (roots, leaves, etc.), M=muck.

[3] Expressed on a dry weight basis.

Hawthorne (Alachua County).

Only one lake had a C/N ratio less than ten (on a weight-weight basis). The total range in C/N ratios was 9.8 to 29.6, but most of the ratios were between 11 and 18. Thus all the lake sediments are relatively enriched in carbon compared to nitrogen. There is a slight trend toward lower C/N ratios in the more eutrophic lakes, but the data are highly scattered. Figure 22 presents a plot of sediment C/N ratio vs. a relative ranking of trophic state (Shannon and Brezonik, 1971b) for the 55 lakes. Similarly a plot of total organic nitrogen vs. total phosphorus in the lake sediments (Figure 23) shows no trend toward a constant ratio; N/P ratios vary from 0.7 to over 40 in these sediments. The "ideal" N/P ratio (by weight) in organisms is about 7 (assuming the frequently cited elemental ratios C:N:P of 105:16:1 by atoms). Most of the sediments have N/P ratios much larger than 7, indicating relative nitrogen enrichment. Thus on this basis alone one would conclude that phosphorus is the more likely limiting nutrient in most Florida lakes. However too much emphasis should not be placed on these figures. The ecological significance of nutrient ratios in sediments remains obscure; certainly the activity or mobility of the species (i.e. their relative abilities for recycling) are more important in determining what is limiting than are static concentration ratios.

The second phase of sediment studies involved evaluation of the rates of nitrogen exchange between sediments and the overlying water. Most of the work on this aspect has been conducted on estuarine sediments (from the Waccasassa Estuary on the Florida Gulf Coast), (Brooks, 1969). Results from a series of ammonia exchange experiments demonstrate the significance of sediments as nutrient sinks and indicate that at least some sediments have a greater tendency to remove nutrients from solution rather than to release them to the overlying water.

An initial experiment with estuarine sediment incubated in aquaria indicated that ammonia levels tended to decline in the overlying water with time whether the aquatic microorganisms were eliminated by various sterilization techniques or not. These preliminary results suggested that these sediments act as a nutrient trap probably by simple sorption onto clays or organic colloids and that from a net viewpoint release of ammonia to the overlying water is unimportant. Several further experiments were conducted to verify this supposition.

Three sediment-water aquaria were set up as closed systems in which any free ammonia evolved as a gas could be trapped and measured. Ammonia free air was bubbled through the water to keep it in slow circulation but the sediments were left undisturbed and developed a sharp sediment-water interface and oxidized microzone in a few days. After the aquaria were equilibrated ammonia was added to two aquaria to raise aqueous concentrations by about 0.1 and 0.5 mg N/l respectively, and concentrations in the water were monitored over a six day period (Figure 24). In the aquarium with no addition, ammonia remained relatively constant, but in both aquaria with added ammonia there was a rapid loss from the water with final values in the vicinity of 0.10 mg N/l. In no case was a significant amount of ammonia stripped from the water;

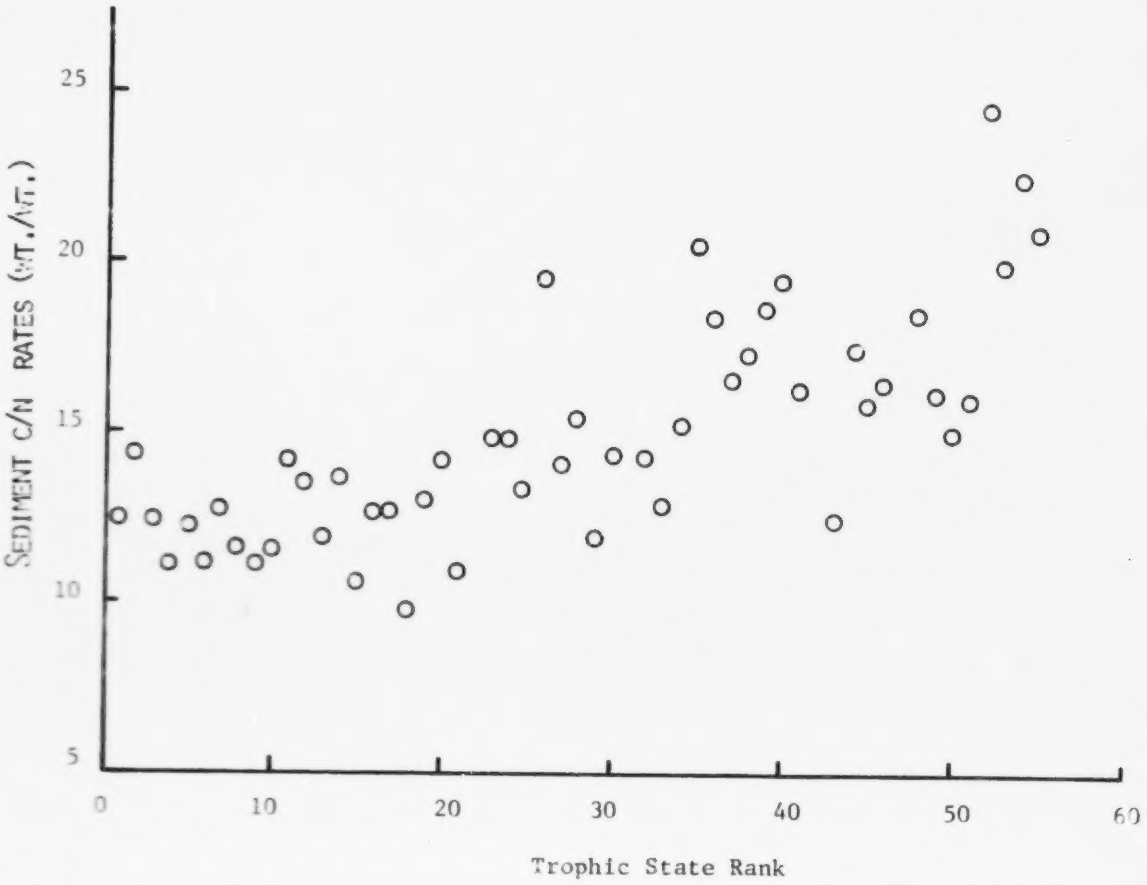

FIGURE 22. CARBON TO NITROGEN RATIO IN 55 LAKE SEDIMENTS AS A FUNCTION OF A RELATIVE TROPHIC STATE RANKING (FROM BREZONIK 1971c). MOST EUTROPHIC LAKE AT LOWEST END OF RANK; MOST OLIGOTROPHIC LAKE AT HIGHEST END.

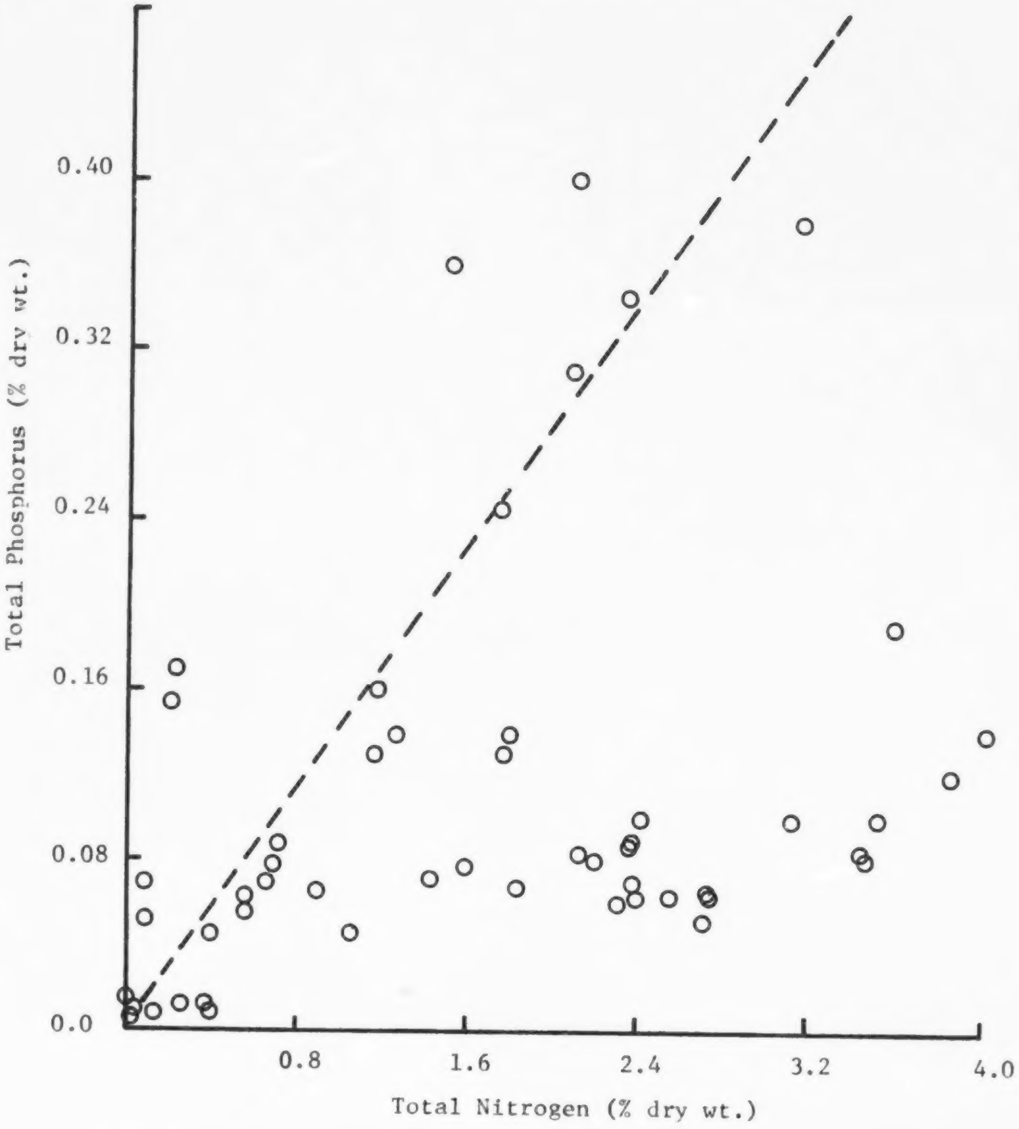

FIGURE 23. TOTAL PHOSPHORUS VS TOTAL NITROGEN IN 55 FLORIDA LAKE SEDIMENTS. DASHED LINE REPRESENTS IDEAL N:P RATIO OF 7:1 (WT./WT.) IN MICROORGANISMS. VALUES BELOW THE LINE INDICATE RELATIVE ENRICHMENT OF N IN SEDIMENT; SEDIMENTS OCCURRING ABOVE THE LINE ARE RELATIVELY ENRICHED IN P.

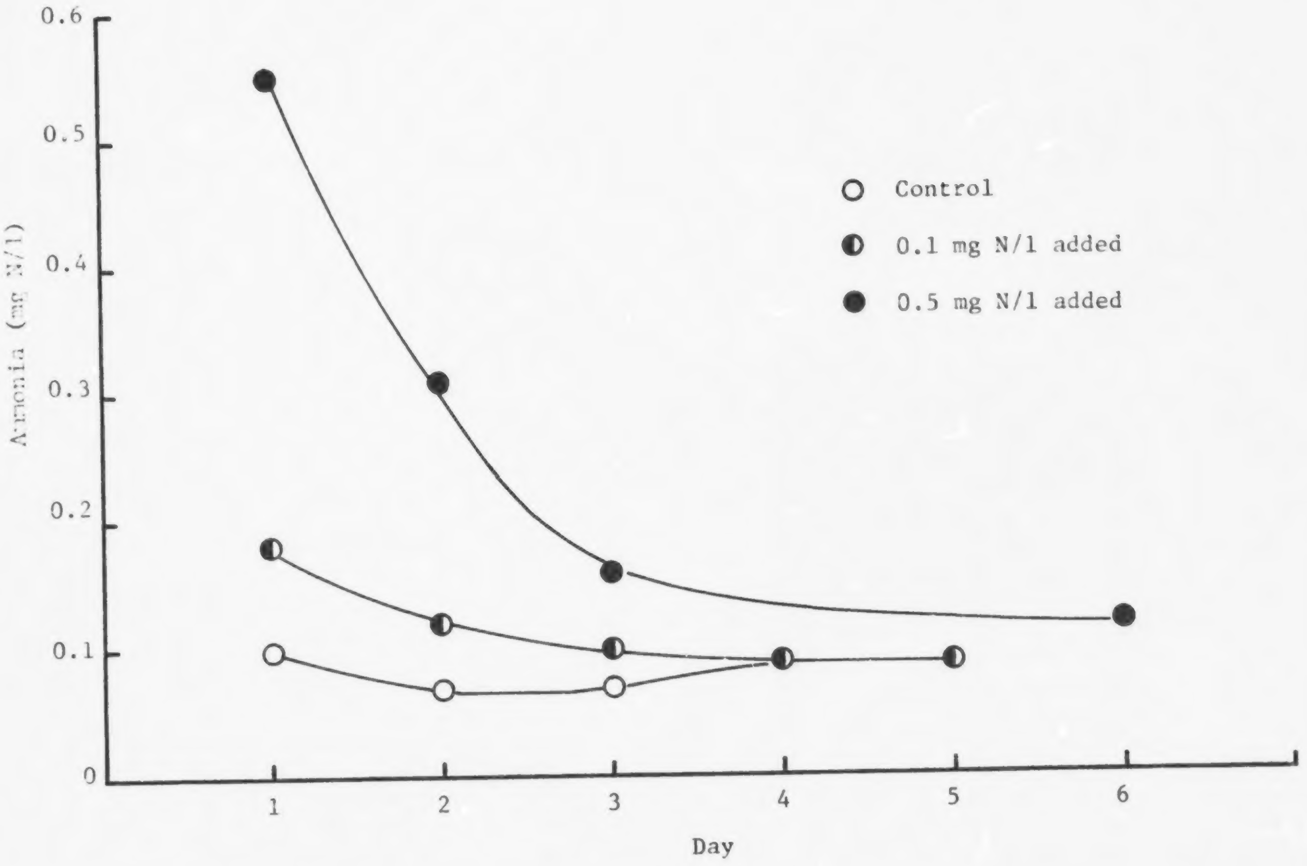

FIGURE 24. UPTAKE OF AMMONIA BY ESTUARINE SEDIMENTS IN AQUARIA. WATER WAS CONTINUALLY MIXED BY BUBBLING AIR BUT SEDIMENTS WERE UNDISTURBED.

the maximum ammonia caught in the acid trap was 5μg as N, and the pH levels in all three aquaria remained in the range 8.0-8.3 throughout the experiment. Aside from sediment uptake, the only likely sink would be assimilation by algae and bacteria. The water was filtered before being added to the aquaria, so initial organism levels were low, and there was no visual evidence for growths either in the water or on the aquaria walls. While not perhaps unequivocal, the results strongly suggest that sediments from the Waccasassa Estuary act to sorb ammonia from solution.

One question raised by the above experiments was whether ammonia uptake by sediments was biologically mediated or a strictly chemical pheomemon. A sediment-water system which was sterilized by cobalt irradiation gave similar rates of ammonia uptake as an unsterilized system did (Figure 25). The results fit neither a first nor second order rate expression but could be approximated by two zero order expressions: a rapid initial uptake (k=1.3mg/l-day) followed by a slower rate (k=0.03 mg/l-day) occurring after several days. This result is reminiscent of the kinetics of phosphate sorption by estuarine sediments reported by Carritt and Goodgal (1954), uptake of pesticides by clays (Haque *et*. *al*., 1968), and sorption of alkyl benzene sulfonates by activated carbon (Weber and Rumer, 1965). In all these cases the rates can be explained by a mechanism involving rapid sorption onto the particle (sediment) surface followed by a slower (rate limiting) intra particle diffusion controlled process.

Several short term ammonia sorption-leaching experiments also indicated the non-biological nature of the phenomenon with rapid uptake of ammonia from the aqueous phase to the sediments. Table 26 summarizes the results from a three hour experiment in which 300 ml. of estuary water was added to four flasks, and 22 g. sediment was added to two of these. An identical amount of ammonium chloride solution was added to one of the pure water and one of the sediment water flasks, and samples were immediately taken from all four flasks for ammonia determinations. The flasks were kept in a constant temperature shaker-bath and replicate analyses were also made after one and three hours. No change was noted in the water only flasks and in the sediment-water flask without added ammonia during the three hours, but a rapid uptake of ammonia occurred in the sediment flask with added ammonia. About ninety-four percent was lost from the water within the three hour period and over sixty percent was sorbed onto the sediment immediately as indicated by the 0 hour concentration of 2.7mg N/l in this flask compared 6.8mg N/l in the flask with added ammonia but without sediment.

The above experiment was also repeated using estuary water from which the background ammonia was removed by boiling and with sediment samples from various depths in a core (interface (top cm), middle (1-10 cm) and the bottom of the core (below 10 cm). In no instance was free ammonia detected in the water phase at the termination of the experiment. Uptake of various ammonia concentrations (1.5 to 4.5 mg N/l) by 10 to 30 g. fresh sediment was also determined using shaken flasks as above with a contact time of 6 h hours. Free ammonia levels in the water after incubation ranged from 0.40 to 1.8 mg N/l, but results did not conform to the Freundlich isotherm. Finally, several experiments were conducted

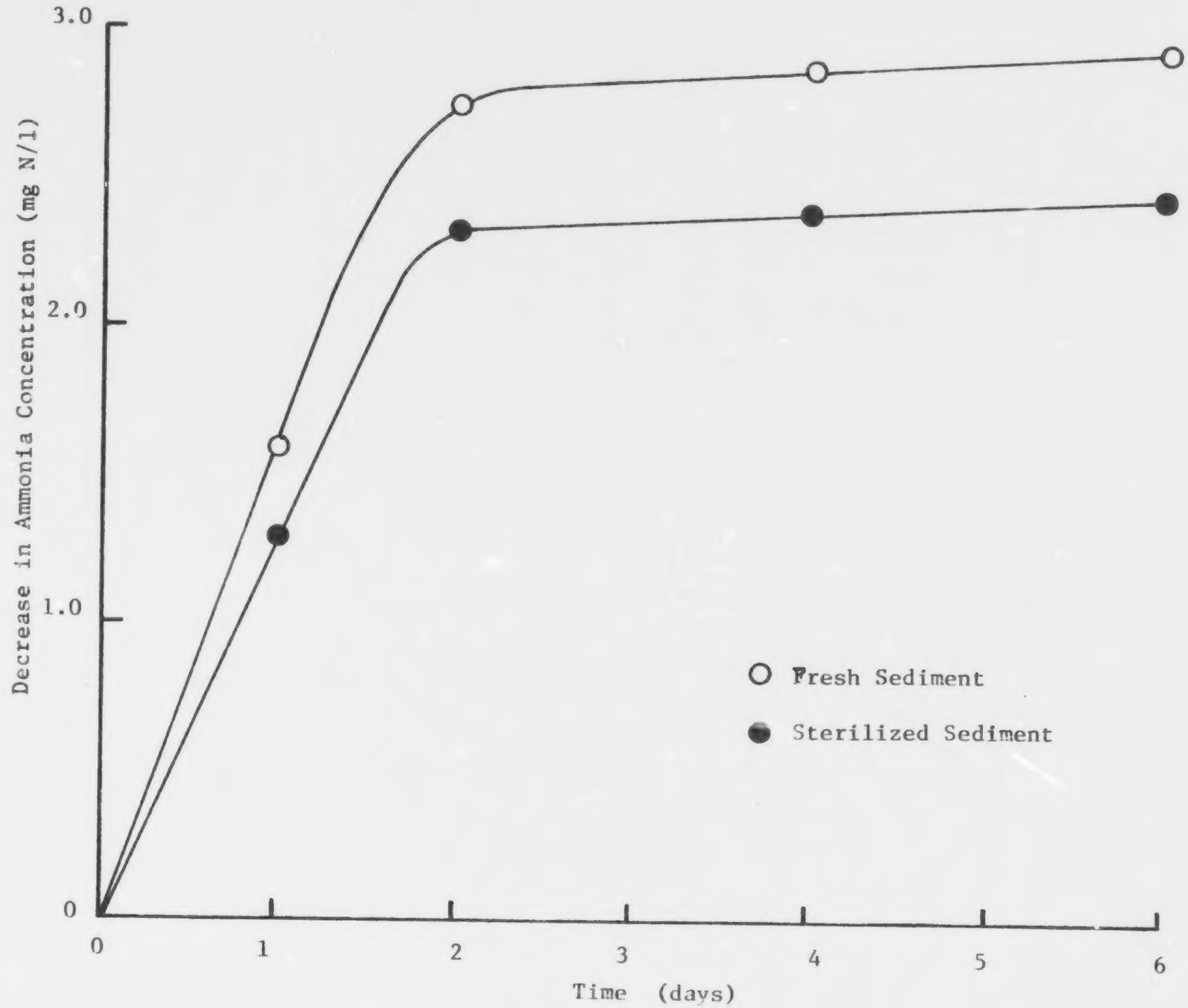

FIGURE 25. DECREASE IN AQUEOUS AMMONIA CONCENTRATION WITH TIME IN AQUARIA WITH FRESH AND STERILIZED ESTUARY SEDIMENT

Table 26.

Uptake of Ammonia by Waccasassa Estuary Sediment in Short-Term Shaker-Flask Experiment[1]

	Estuary Water[2]	Estuary Water with Added NH_4Cl[2]
Control (no sediment)	0.11	6.80
5 min	0.11	2.70
1 hr	0.11	1.06
3 hr	0.10	0.35

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[1] From Brooks (1969). Four flasks had 300 ml estuary water added. Ammonium chloride solution was added to 2 flasks in identical amounts and 22 g fresh sediment was added to one of these. Of the 2 remaining flasks, one was a control and the other had 22 g sediment added to it.

[2] Ammonia concentrations in mg N/l; controls (no sediment) remained constant throughout the experiment.

with blended sediments. In these cases as much as 0.34 mg NH_3-N could be obtained per gram (dry weight) of freshly homogenized sediment.

Ammonia within soil (and presumably) sediment exists both in a form exchangeable by other cations such as sodium, calcium or potassium and in the fixed or non-exchangeable state (Bremner, 1965; Jackson, 1958). In an estuarine environment with high concentrations of sodium, magnesium, potassium and calcium present, it might be predicted that exchangeable ammonia on the sediments would be small, and the absorbed ammonia might be present in the non-exchangeable form. This agrees with the above results in which little tendency for ammonia leaching was observed. However it should be noted that the concentrations of sodium and other cations in a marine or estuarine environment do not approach the levels used in standard soil chemistry to displace exchangeable ammonia (Jackson, 1958). Bremner (1965) has indicated that grinding of sediments would release a portion of the fixed ammonia. This was found to be the case with Waccasassa Estuary sediments homogenized in a Waring blender. Probably some of this ammonia resulted from disruption of cellular material.

The above experiments indicate that ammonia uptake and release by Waccasassa Estuary sediments is largely controlled by strictly chemical (sorption) phenomena. X-ray diffraction analysis of the sediment showed the presence of small amounts of clays, primarily of a kaolinitic nature, but further experiments are necessary to describe this sorption mathemetically.

Rapid losses of ammonia from solution have also been found with lake sediments in some preliminary experiments. Figure 26 shows the ammonia remaining in solution vs. amount of ammonia added to sediment (dry weight) from Bivin's Arm, a highly eutrophic lake near Gainesville, Florida. In this experiment 10 ml of fresh sediment, equivalent to 0.4 g. dry weight, was suspended in 100 ml demineralized water in 125 ml Erlenmeyer flasks, which were continuously shaken on a shaker table for 2 hours. At low concentrations of added ammonia the levels after incubation were higher than the added levels; for example at 0.1 mg N added 0.45 mg N (both per 100 ml sample) was measured in solution after incubation. But at higher levels of added ammonia the amounts left were relatively small (e.g. with 5.0 mg N added 0.92 mg N remained in the 100 ml sample). These results can be interpreted more readily in light of Figure 27 which plots the amount of ammonia leached from sediment vs. the amount of sediment added to 100 ml of deionized water. Thus, with 0.4 g. (dry weight) sediment suspended in 100 ml of water, 0.37 mg ammonia N is released, but the amount of leaching is highly dependent on the sediment /water ratio. At 2.0 g. (dry weight) per 100 ml of water (a 5-fold increase) only 0.65 mg ammonia N is released (a less than 2-fold increase). These results seem to suggest that sediments from Bivin's Arm act as a buffer to maintain a certain level of ammonia in their overlying

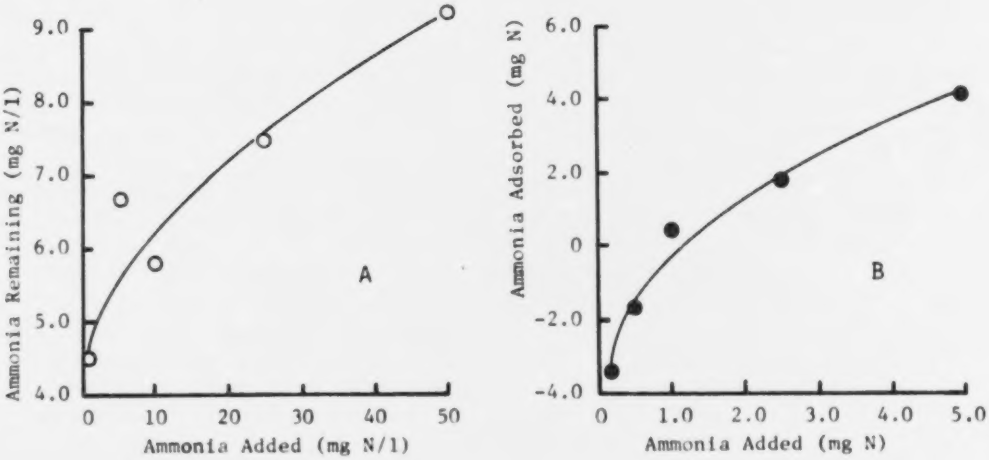


FIGURE 26. AMMONIA UPTAKE BY LAKE SEDIMENT: (A) AMMONIA CONCENTRATION REMAINING AFTER 2 HOURS INCUBATION AS FUNCTION OF INITIAL AQUEOUS CONCENTRATION; (B) NET SORPTION OF AMMONIA (AMOUNT ADDED MINUS AMOUNT REMAINING) VS INITIAL AMOUNT OF AMMONIA ADDED TO WATER

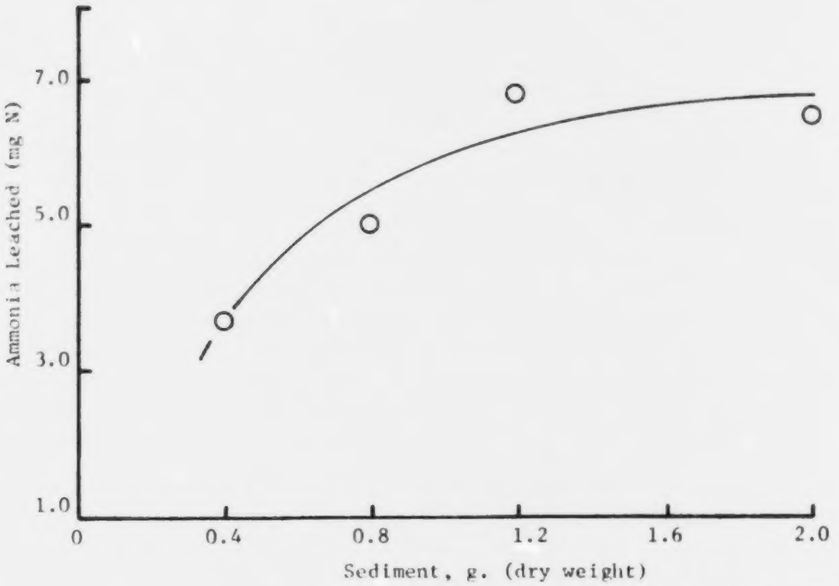


FIGURE 27. AMMONIA LEACHED AS A FUNCTION OF AMOUNT OF LAKE SEDIMENT ADDED TO 100 ML DEIONIZED WATER

(or interstitial) waters. When water low or entirely depleted in ammonia is placed in contact with these sediments, they tend to release ammonia to the water, but when more concentrated ammonia solutions are contacted with the sediments they sorb the ammonia decreasing solution concentration toward some equilibrium value.

Obviously much remains to be done to characterize the equilibria and kinetics of ammonia exchange between sediments and water. However, the above experiments indicate that rapid and large exchanges apparently mediated by simple sorption phenomena are possible for nitrogen as well as for phosphorus. While the nitrogen cycle within the water column is nearly exclusively a biochemical phenomenon, non-enzymatic processes may be more important within sediments and at their interface with water.

While sediments can act as either in situ sources or sinks, denitrification acts solely as a sink. As described in Section IV, this reaction occurs in water when oxygen is depleted and is mediated by facultative bacteria who use nitrate as a terminal electron acceptor (in the place of O_2) with the major reduction product being N_2. However, varying amounts of ammonia and organic nitrogen are apparently always produced in situ simultaneous with denitrification, as has been shown by a number of investigations (see Section IV) including one supported by this project (Tang, 1968). In the latter study from 70 to 80 percent of added nitrate nitrogen was recovered as N_2 in laboratory sludge digesters, and the remainder was reduced to ammonia and organic nitrogen. This phenomenon occurred even at high ammonia levels where nitrate assimilation would seem a superfluous nitrogen source. It is not possible to ascertain from such mixed culture experiments as these and others (Brezonik and Lee, 1968; Goering, 1968; and Keeney et al., 1971) whether the biological agents of denitrification are the same as those reducing nitrate to the level of ammonia, but from an ecological viewpoint this is perhaps unimportant. What is important however is that both reactions occur whenever oxygen is depleted in a nitrate containing environment. Thus denitrification (i.e. nitrogen lost to the system) is not equivalent to nitrate lost and any attempts to assess the significance of denitrification as a nitrogen sink must take this into account.

Denitrification in Florida lakes would seem to be of minor importance although there may be unique situations where this statement is not valid. In the study of 55 lakes in north central Florida (Brezonik, 1971c) only about 8-9 of them appear to develop stable summer stratification, and of these only 4 develop anoxic conditions in a significant volume of their hypolimnia. In addition several other lakes develop temporary stratification and lose their oxygen in the bottom half to one meter, probably because of a high sediment oxygen demand. Denitrification would be possible in these regions; however from a quantitative point of view they represent only a minor fraction of the water volume in the lakes. Further nitrate concentrations in Florida lakes are typically low. Most of the waters are soft and acidic, and nitrification is not favored by such environments. Hence most of the inorganic nitrogen in Florida lake waters occurs as ammonia. In those lakes which stratify nitrate levels greater than 0.10 mg N/1 are rare.

Thus, denitrification does not appear to be a significant nitrogen sink in Florida lakes and it was felt that a detailed evaluation of its occurrence was not worth pursuing. On the other hand, denitrification may be important in areas of Florida where nitrate rich ground water seeps through organic and anaerobic sediments or where surface drainage enriched in nitrate from agricultural fertilization passes through anoxic soils or sediments. However, this aspect was beyond the scope of the present project.

SECTION IX

ANALYTICAL INVESTIGATIONS

While research on the analytical aspects of nitrogen in natural waters was not a primary function of this project, some work was undertaken to evaluate procedures used in the main phases of this research. Because the acetylene reduction method was used extensively in the nitrogen fixation studies and since this is a relatively new method (first reported by Stewart *et*. *al*., 1967), an evaluation of the procedure was considered essential. Also, a large number of inorganic nitrogen (ammonia and nitrate) analyses of natural waters were performed during this project and also in conjunction with other investigations on lake eutrophication conducted in this laboratory. A number of difficulties were encountered with the usual automated procedures for these constituents, especially interferences arising from the high color content of many Florida lakes. Consequently a study of these procedures was undertaken to devise methods of overcoming these interferences. The acetylene reduction assay relies on the fact that acetylene acts as a competitive inhibitor of nitrogen with the enzyme nitrogenase, which reduces it to ethylene. Production of the latter gas can be quantified by flame ionization gas chromatography, and the method, though an indirect assay, is rapid, highly sensitive, and inexpensive. Laboratory experiments were conducted to determine optimum incubation conditions, to evaluate the method as an indirect assay for nitrogen fixation, and to study environmental conditions affecting nitrogen fixation by blue-green algae.

The basic procedures used for acetylene reduction assays of sediments and waters are described in Appendix A. Figure 28 shows a typical chromatogram obtained from one cc. of gas phase in an incubated sample. The column of Poropak R cleanly separated ethylene from any traces of ethane (sometimes present in the acetylene) and from the large peak of acetylene. Early in the development of this technique for assays of low fixation rates in sediments it was recognized that controls were necessary with each set of samples. These consisted of samples carried through the basic sequence (Appendix A) except that 1 ml of 50 percent trichloroacetic acid was added before inserting the acetylene. Controls were necessary because rather wide variations of background ethylene occurred in the "purified" acetylene used. Ethylene levels were especially high in full tanks, but contaminant levels declined with use. In order to avoid complications arising from very high backgrounds it was found expedient to waste about the first third of a tank.

Sediments for these evaluation studies were obtained from the Waccasassa Estuary (Gulf Coast of Florida) and from Lake Kanapaha, near Gainesville, Florida. Water samples for this phase were obtained from

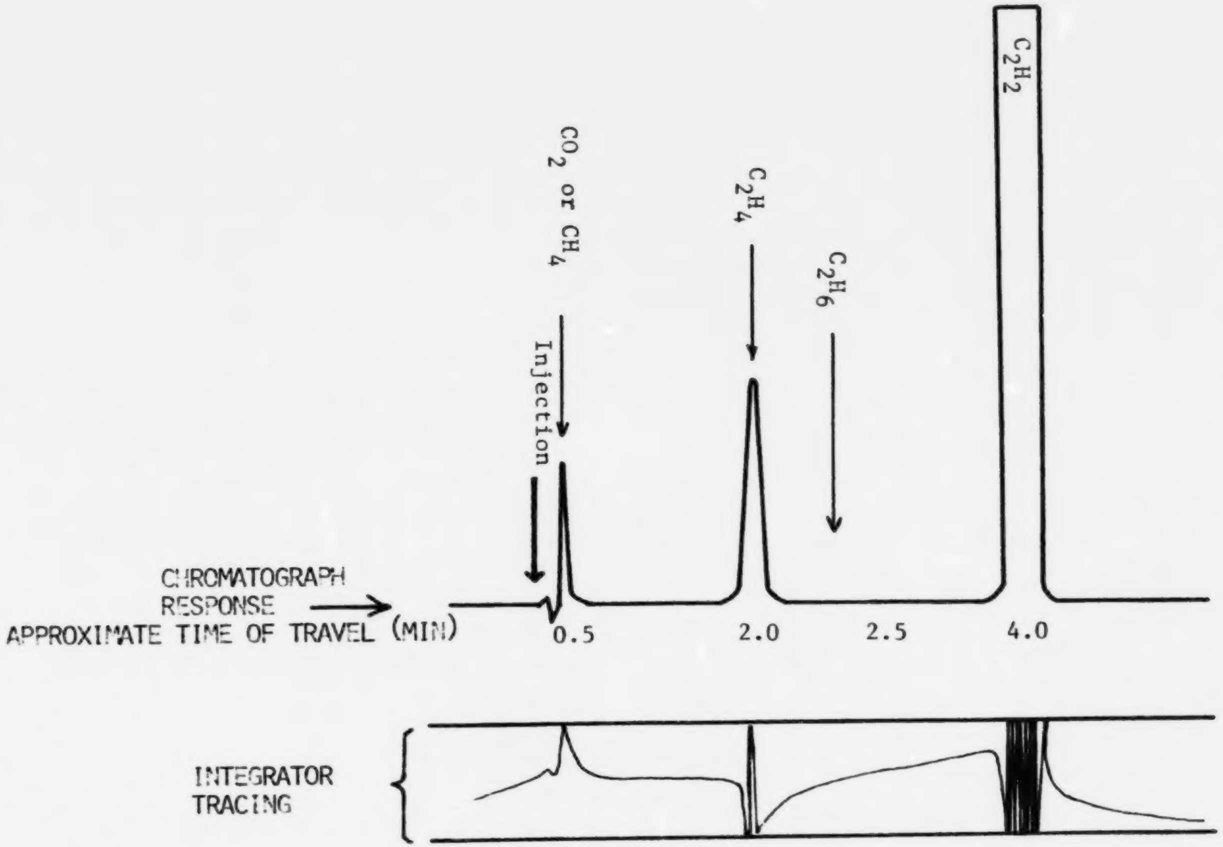


FIGURE 28. TYPICAL CHROMATOGRAM ILLUSTRATING IDENTIFICATION OF PEAKS AND MEASUREMENT OF PEAK AREA FOR ACETYLENE REDUCTION ASSAY.

a small pond on the University of Florida golf course, which conveniently has a dense bloom of Anabaena (2 species) throughout the summer. Table 27 presents replicate data for 3 pond samples incubated for one hour and 8 samples incubated for 2 hours under laboratory conditions. Mean C_2H_4 production from the two hour incubations was 51.0 ± 1.9 nmoles C_2H_4/mg N-hr. at the 95% confidence level. The mean one hour incubation rate was 84.2 nmoles/mg N-hr. The replication is obviously within acceptable limits for biological phenomena. However, the rates decreased rather significantly with longer incubation times, probably because of bottle effects. Possibly, removal of N_2 from the organisms causes nitrogen starvation over this period of time. Table 28 presents results of a replication using Waccasassa Estuary sediments. A mean of 11.5 ng. ethylene per g. dry wt. sediment-hr. with a relative standard deviation of 3.6 percent was found for six replicates, again indicating the methodogy has adequate precision. Figure 29 shows the effect of incubation time on the amount of ethylene produced by estuary sediments. Up to about one hour the rate appears linear, but after that it drops markedly. Thus the efficacy of short incubations is apparent.

The effect of light and the presence of N_2 on the time course of acetylene reduction was also studied on water from Golf Course Pond. One set of samples was purged with the O_2-CO_2-Ar mixture to remove N_2; another set was left unpurged. A set of purged and unpurged samples were each incubated in the light and in the dark for times ranging from 0.5 to 3 hours (Figure 30). Correlation of nitrogen fixation rates with primary production is evident from the much higher rates of ethylene production in the light than in the dark. However, fixation is evidently not completely dependent on photosynthesis to supply reduced hydrogen ($NADPH_2$) for fixation; otherwise fixation would have ceased nearly immediately after light was removed. Reduced hydrogen apparently is also supplied by respiration (oxidation of organic compounds). In all cases, the rate of ethylene production decreased with longer incubation times but the effect was most dramatic in the light incubated, unpurged set. Rates were linear for about an hour in the light and for about the same time in the dark although variability in the dark incubation data do not permit great accuracy in this conclusion. The decline in production with time could result from bottle effects, nitrogen starvation (in purged samples), organism shock from manipulation or from incubation conditions (since temperature and light conditions in the lab differed from the natural conditions). Removal of N_2 did not significantly affect the rates measured in dark incubations, but these rates were low and not as precise. In the light, the presence of N_2 has a depressant effect, especially over longer incubation times. However the magnitude of the depression is not too large to preclude the direct addition of acetylene to unpurged samples if field conditions prevent purging. Based on Figure 30 results obtained in this manner would be 25 to 30 percent too low (assuming incubations of 0.5 to 1 hour) but this error may be acceptable in surveys or in difficult field circumstances where transport of purging tanks would be cumbersome or otherwise undesirable.

The effect of N_2 on acetylene reduction was studied in greater detail in a further experiment on Golf Course Pond water. Since C_2H_2 is

Table 27. Replicate Data on Nitrogen Fixation by Natural Population of *Anabaena sp*. from Golf Course Pond

Sample No.	Incubation Time (Hours)	C_2H_4 Produced Nanomoles	Fixation Rate nmoles C_2H_4/mg N-hr
1	1	12.0	85.7
2	1	11.2	86.2
3	1	9.7	80.8
4	2	11.6	48.3
5	2	13.7	48.9
6	2	13.6	52.3
7	2	16.6	55.3
8	2	16.9	52.8
9	2	16.9	52.8
10	2	12.8	53.3
11	2	15.6	52.0

[1] See text for details of incubation and statistical analysis of results.

Table 28. Replication of Acetylene Reduction by Estuary Sediments

Sample Dry Weight g.	Ethylene Nanomoles	Production nM/g. dry wt.
4.827	2.00	0.414
4.631	1.83	0.396
4.971	2.16	0.436
4.961	2.08	0.418
4.582	1.83	0.400
4.760	1.91	0.403

$\bar{x} = 0.410$

$s^2 = 0.0062$

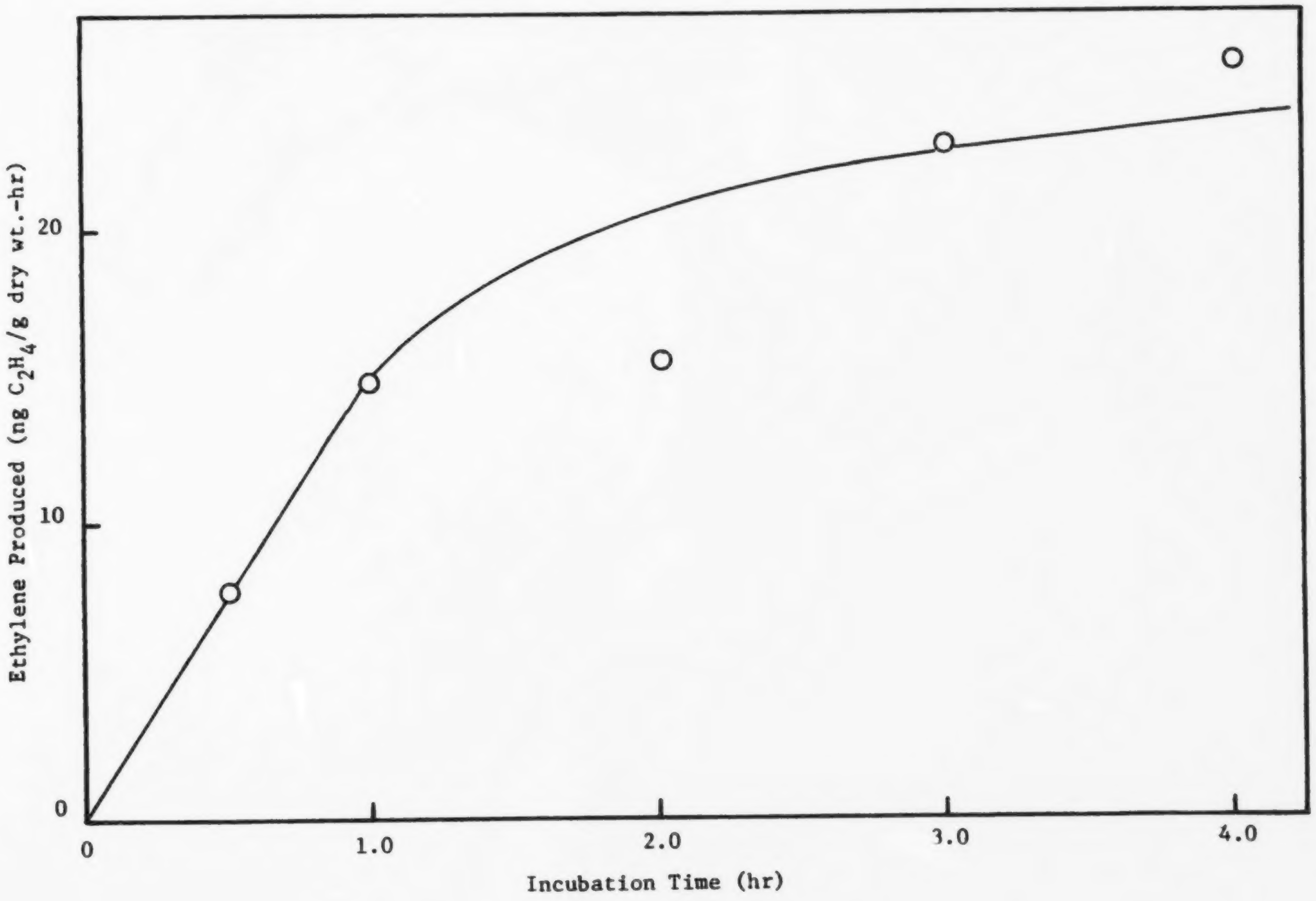


FIGURE 29. ETHYLENE PRODUCTION VS. TIME FOR WACCASASSA ESTUARY SEDIMENTS

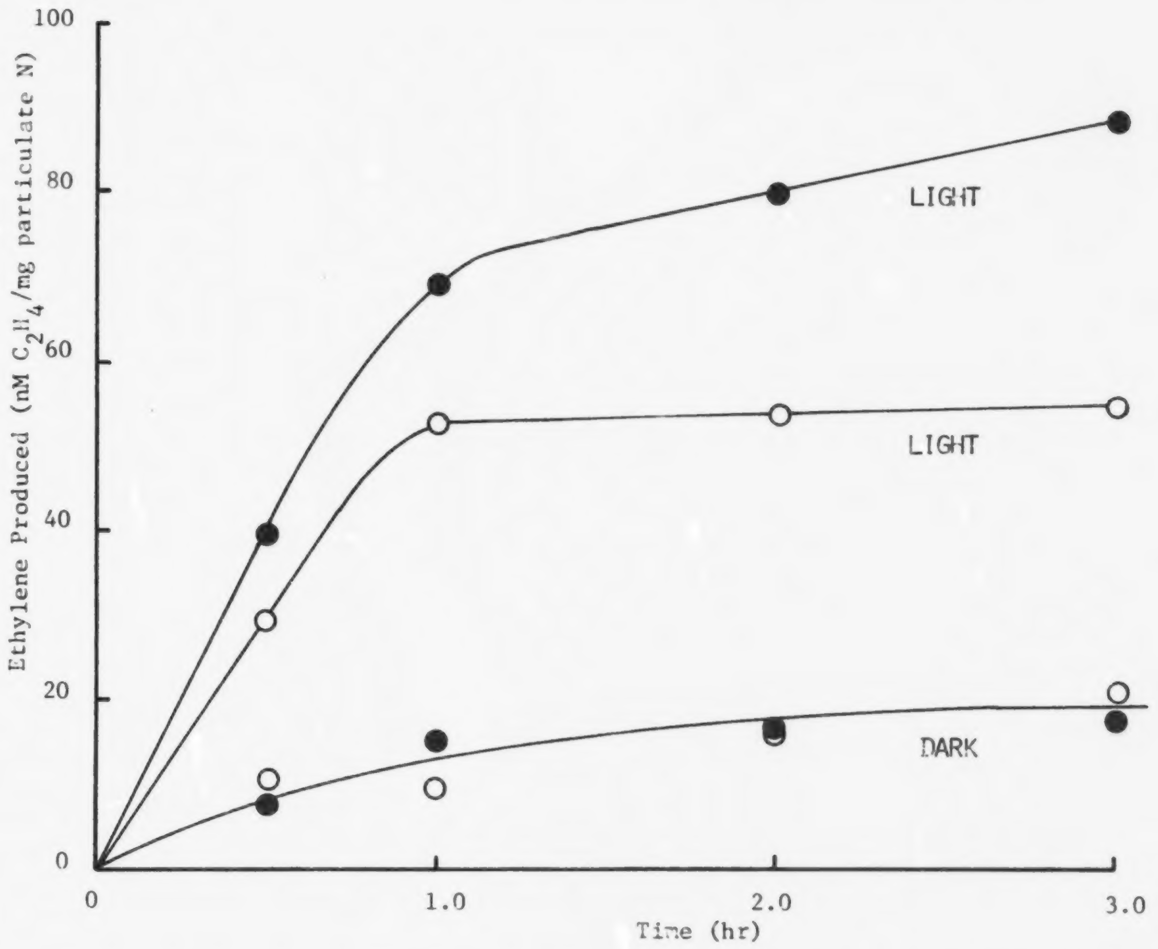


FIGURE 30. TIME COURSE OF ACETYLENE REDUCTION BY NATURAL ANABAENA POPULATION UNDER CONDITIONS OF LIGHT AND DARK IN THE PRESENCE OF 0.78 ATM N_2 (OPEN CIRCLES) AND IN THE ABSENCE OF N_2 (CLOSED CIRCLES).

apparently competitive inhibitor of N_2, the converse should be true, and a reciprocal (Lineweaver-Burk) plot of reaction velocity vs. substrate concentration should give a competitive inhibition pattern (Figure 31). This pattern was found for the natural Anabaena population in the pond (Figure 32). In this experiment varying amounts (0.5, 1.0, 3.0, 5.0 ml) of acetylene (the substrate) were added to serum bottles with 25 ml of pond water both in the presence of inhibitor N_2 (0.78 atm) and its absence (by purging air from the bottles with the O_2-CO_2-Ar gas mixture). Samples were incubated for one hour in the light at 22°C. Competitive inhibition patterns (Cleland, 1963) are given by inhibitors that combine with or react at the same active site on the enzyme as the normal substrate. The molecular similarity of N_2 and C_2H_2 suggests they would react at the same site on nitrogenase and that each should thus competitively inhibit reaction of the other. Schollhörn and Burris (1966, 1967) found competitive inhibition of N_2 reduction by acetylene, but Burris (1969) reported that Hwang and Burris (1968) did not.

Differentiating between competitive inhibition and other patterns (e.g. non-competitive inhibition) is not always a simple or unequivocal matter. The distinguishing feature of the former pattern is that the reciprocal plots for different inhibitor levels intersect on the ordinate; in the latter pattern the plots intersect to the left of the ordinate. The physiological significance of this is that in competitive inhibition the maximum reaction velocity (V_{max}) is unaffected by the presence of inhibitor. V_{max} is obtained from the y-intercept of the 1/v vs. 1/S curve on a Lineweaver-Burk plot; at $1/S = 0$, $S = \infty$ or in physical terms S is at a saturating concentration and v is at a maximum level ($\equiv V_{max}$). Since the y-intercept is unaffected by inhibitor in competitive inhibition (see Figure 31), V_{max} is unaffected, meaning that very high (saturating) levels of substrate can completely overcome inhibitor effects. If inhibitors change the Lineweaver-Burk plot y-intercept values, V_{max} is changed, and even saturating levels of substrate cannot completely overcome inhibitor effects. Note however, that placement of lines through 1/v vs. 1/S data is subject to a certain amount of experimental error. Whether or not curves for varying inhibitor levels actually intersect exactly on the y-intercept is always subject to some uncertainty, the magnitude of which increases with scatter of the data.

What is perhaps more significant about Figure 32 than whether the data yield an exact pattern of competitive inhibition is that acetylene reduction rates can be described by Michaelis-Menten kinetics since the reciprocal (Lineweaver-Burk) plots are linear (within experimental error). This fact supports the enzymatic basis for acetylene reduction, and the fact that N_2 inhibits (in some way) the rate of acetylene reduction is further evidence that ethylene production from acetylene is mediated by nitrogenase and is therefore an indirect assay of nitrogen fixation. There is at present no evidence in the literature for the production of ethylene from acetylene by any non-nitrogen fixing organisms; however the possibility that such organisms might exist should be kept in mind. These techniques (i.e. determination of N_2 inhibition and of fit to enzyme (Lineweaver-Burk or Michaelis Menten)

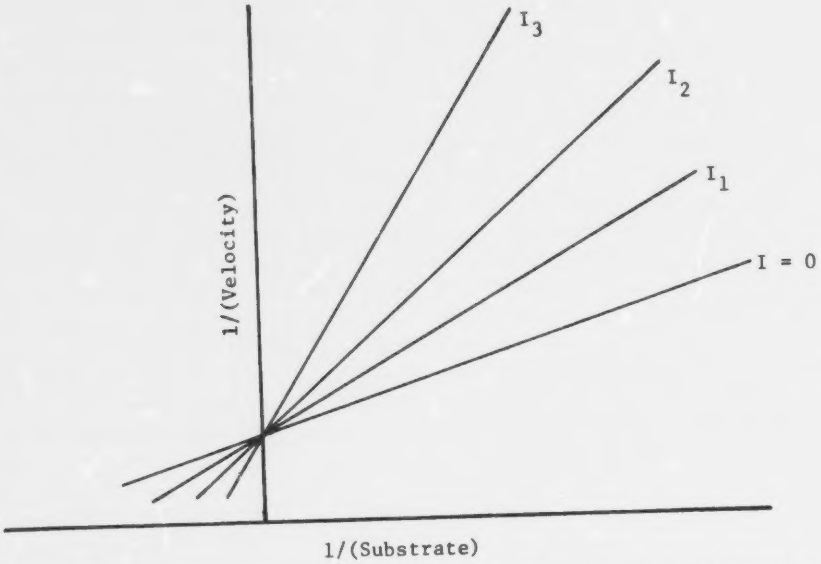


FIGURE 31. THEORETICAL LINEWEAVER-BURK PLOT FOR COMPETITIVE INHIBITION OF ENZYME REACTION RATE, WHERE I_1, I_2, I_3 REPRESENT INCREASING INHIBITOR LEVELS.

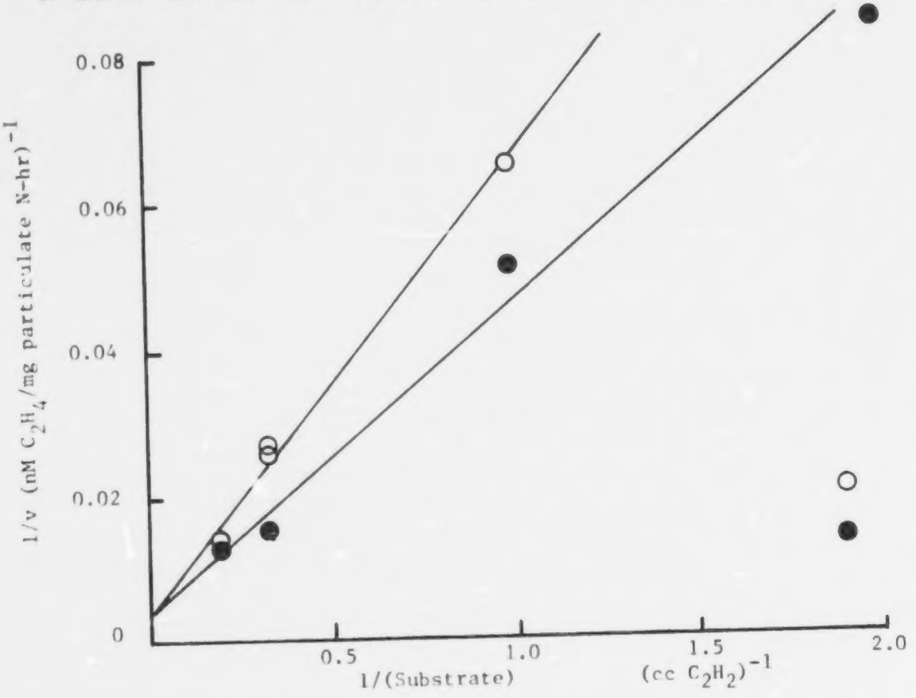


FIGURE 32. COMPETITIVE INHIBITION OF ACETYLENE REDUCTION IN NATURAL ANABAENA SP. POPULATION BY N_2.

kinetics) should be useful in relating ethylene production in an environmental sample to nitrogen fixation, and they were used for this purpose in the studies of fixation in sediments (Section VII).

As a further evaluation of the acetylene reduction method, the effects of two other nitrogenase inhibitors on C_2H_4 production were studied with Golf Course Pond water containing Anabaena sp. Both carbon monoxide and nitrous oxide are reported to inhibit nitrogen fixation. Presumably, they should also inhibit nitrogenase reduction of C_2H_2 to C_2H_4. In one experiment varying amounts of C_2H_2 and CO were added to pond samples and incubated in the light for one hour. The results (Figure 33) indicate that CO is a very potent inhibitor of acetylene reduction, and the data seem to fit a Lineweaver-Burk plot (Figure 34). However, for some inexplicable reason the curves for various CO levels intersect to the right of the y-axis. The author is unaware of any enzyme kinetic expressions yielding this pattern, and this aspect of the result would seem an artifact. Schöllhorn and Burris (1966, 1967) found competitive inhibition of C_2H_2 reduction by CO, but Hwang and Burris (1968) did not. Perhaps the intact organism systems are too complex to consistently yield the simple, pure enzyme inhibition patterns. The inhibition of C_2H_2 reduction by CO is good evidence for the biochemical nature of the reduction, but the data are inconclusive regarding the nature of the enzyme inhibition pattern and hence the mechanism of inhibition.

In another experiment, samples of Bivin's Arm lake water were purged to remove N_2, and varying amounts of C_2H_2 and N_2O were added. The results indicated a typical Michaelis-Menten response to added C_2H_2, but no significant inhibition by N_2O was found in the range 0.04 to 0.20 atm (Figure 35). There is no information in the literature on the range and nature of N_2O inhibition of nitrogenase, but Hardy et al. (1968) reviewed other studies showing that nitrogenase reduces N_2O to N_2. Possibly N_2O is much more loosely bound by nitrogenase than is C_2H_2, so that even at the lowest C_2H_2 concentration and highest N_2O concentration, C_2H_2 may essentially saturate the enzyme active site, at least in comparison to N_2O. The lack of inhibition by N_2O cannot be explained by solubility differences, for N_2O is somewhat more soluble than C_2H_2, respective Henry's Law constants at 20°C being 1.98×10^{-3} and 1.21×10^{-3}. Regardless of the explanation for these results, N_2O would not appear useful in inhibition studies with nitrogenase.

It is well known that nitrogenase is an adaptive enzyme and that cells fix nitrogen only when other available sources (NH_3, NO_3^-) are depleted. In this sense, ammonia can be considered to inhibit fixation. The question arises whether ammonia inhibits the activity of existing nitrogenase or represses synthesis of new enzyme. Another pertinent question concerns the level of ammonia which produces enzyme inhibition or repression of enzyme synthesis. Recent data by Hardy et al. (1968) indicates that ammonia does not directly inhibit fixation but merely represses synthesis of nitrogenase enzyme. According to Stewart (1968) levels of ammonia in the environment are unlikely to be high enough to inhibit fixation immediately, but the quantitative aspects of this question are poorly understood. The immediate and longer term effect

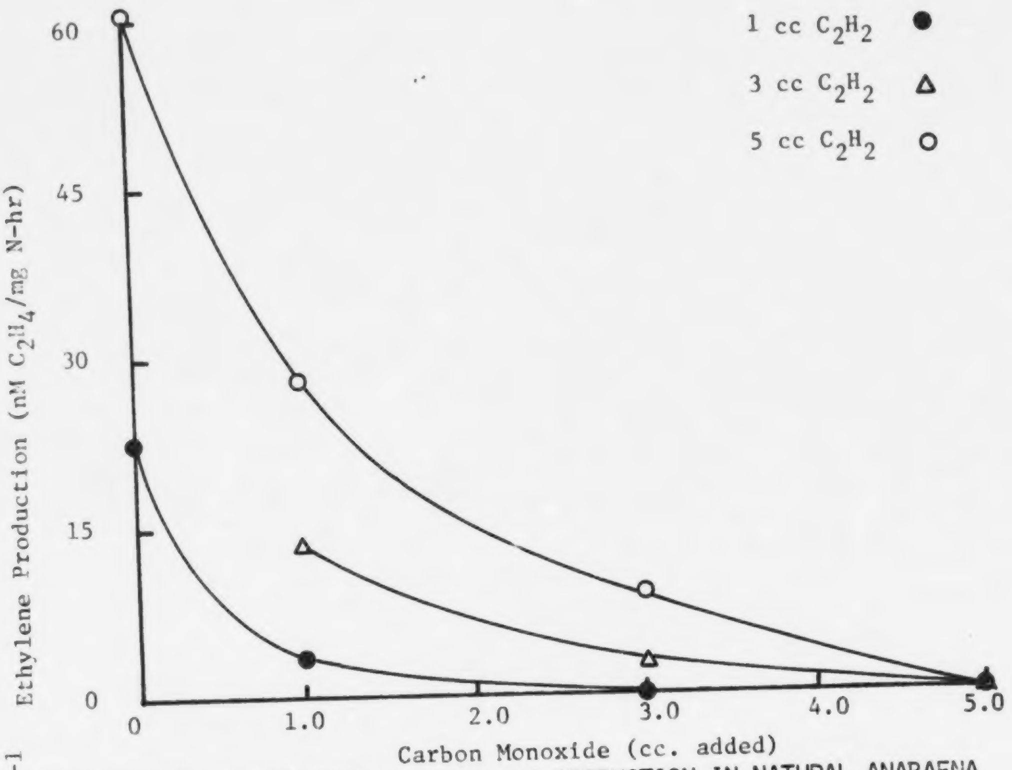


FIGURE 33. INHIBITION OF ETHYLENE PRODUCTION IN NATURAL ANABAENA POPULATION BY CARBON MONOXIDE AT SEVERAL LEVELS OF SUBSTRATE (ACETYLENE)

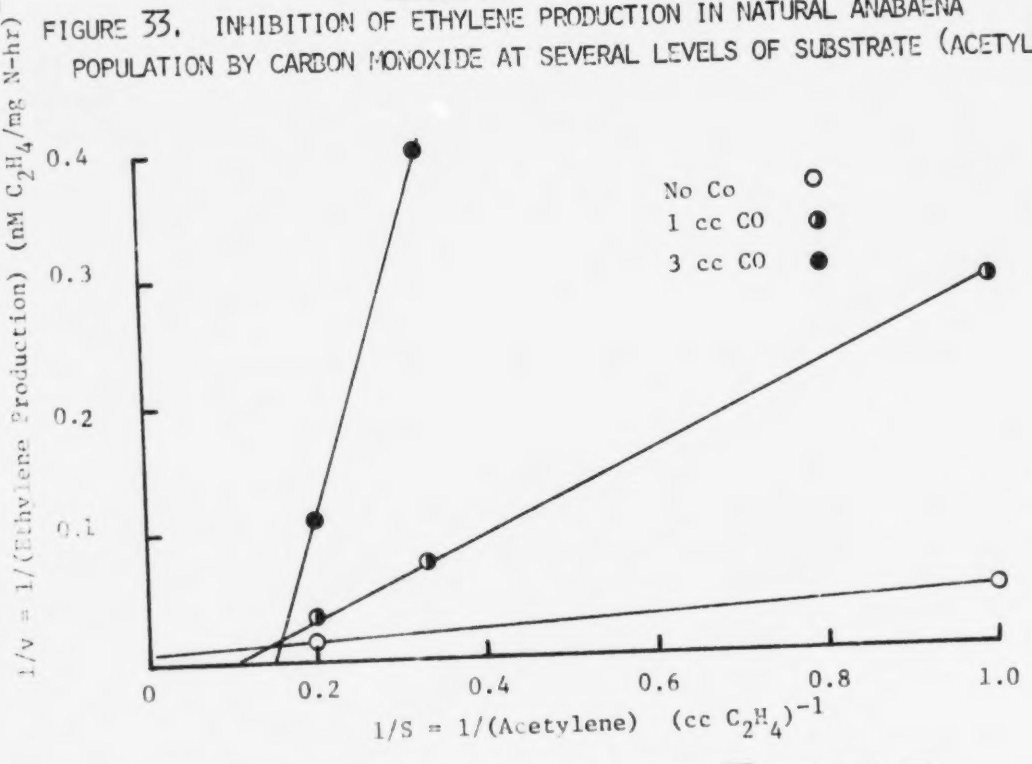


FIGURE 34. REPLOT OF DATA FROM FIGURE 33 ACCORDING TO LINEWEAVER-BURK EXPRESSION.

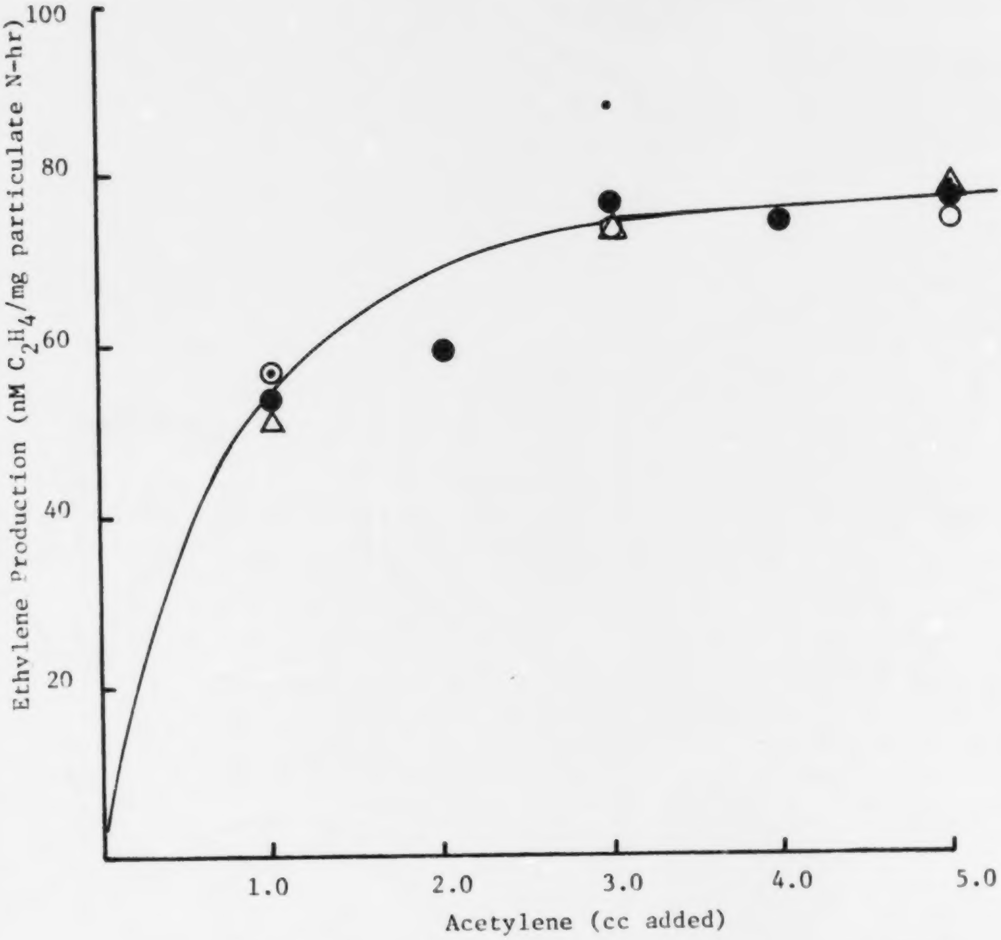


FIGURE 35. ETHYLENE PRODUCTION RATES FOR NATURAL ANABAENA POPULATION VS ACETYLENE AT FOUR LEVELS OF NITROUS OXIDE (N_2O) SHOW NO SIGNIFICANT INHIBITION. SYMBOLS: CLOSED CIRCLES, 0 ATM N_2O; OPEN CIRCLES, 0.04 ATM N_2O; DOTS, 0.12 ATM N_2O; TRIANGLES, 0.20 ATM N_2O.

of ammonia on nitrogen fixation by a natural Anabaena population (from Golf Course Pond) was studied in an attempt to clarify the matter. Two liter samples from the pond were inoculated with 0.10 and 0.50 mg NH_3-N/1 and one control (no ammonia added) was also set up. Samples were taken from each bottle for acetylene reduction assay within 20 minutes of ammonia addition, and a second set of samples were assayed after the bottles had been incubated in situ for 24 hours. The pond had an especially heavy bloom of Anabaena and Microcystis species at this time. Samples for ammonia analysis were taken initially, immediately after ammonia addition and at the end of 24 hours. Results from this experiment are presented in Table 29 and Figures 36 and 37. Ammonia (up to 0.5 mg N/1) has no direct inhibitory effect on C_2H_2 reduction; i.e. the initial samples showed no decrease in C_2H_4 formation with increasing ammonia concentration. There was a depression in C_2H_4 production possibly related to ammonia concentration after 24 hours incubation; however, the control bottle also showed a lower rate after the twenty-four hour incubation. This was probably caused by bottle "wall effects," e.g. death of some N fixing organisms. Furthermore the differences in acetylene reduction between the control and ammonia enriched samples after 24 hours were much larger (on a reltive basis) for the lowest acetylene concentrations (1cc/bottle) than for the highest acetylene levels. Experimental errors tend to be lower at higher activities and higher substrate levels, so more weight should be put on these values. Lineweaver-Burk plots tend to emphasise the differences found at lowest rates and substrate levels, where precision is poorest, and in this sense such plots can be misleading. Thus the effect of ammonia on nitrogen fixation after 24 hours is somewhat ambiguous. A slight depression seems evident, even considering the above cautionary comments, but the difference in rates between the control and the sample with 0.5 mg N/1 added was only about 8 percent (for the 5cc acetylene samples). These results therefore suggest that ambient ammonia levels (which in lakes are infrequently higher than 0.5 mg NH_3-N/1) should not directly influence nitrogen fixation rates at least over short periods of time.

The second analytical study performed in relation to this project concerned the evaluation of automated inorganic nitrogen methodologies, particularly in reference to interferences from organic color and dissolved free amino acids. As part of our investigations of trophic conditions in Florida lakes (Brezonik, 1971c) and in relation to the studies of nitrogen in these lakes conducted as part of this project, a large number of ammonia, nitrite and nitrate analyses have been performed in this laboratory over the past several years. Because of the volume of work involved, analyses were performed by automated (Technicon Auto Analyzer) techniques. In the nitrite and nitrate procedures chemistries employed in Standard Methods (A.P.H.A., 1965)(i.e., the diazotization of sulfanilic acid and α-naphthylamine by nitrous acid to form a pink dye and the well-known brucine method for nitrate) were automated directly.

The Standard Methods procedures for ammonia are either inappropriate for the waters being analyzed(viz. direct Nesslerization) or are unsuitable for automation (viz. distillation and Nesslerization or titra-

Table 29. Ammonia Inhibition of Nitrogenase in a Natural Population of *Anabaena sp.*

	Initial Effect			Effect After 24 Hours		
Ammonia Added[1]	Measured Ammonia[1]	C_2H_2 Added[2]	Production Rate of C_2H_4[3]	Measured Ammonia[1]	C_2H_2 Added[2]	Production Rate of C_2H_4[3]
None (Control)	0.00	0.5	19.2	0.03	1.0	2.40
		1.0	34.2		3.0	5.28
		3.0	102		3.0	58.0
		5.0	111		5.0	67.8
0.1	0.09	1.0	33.9	0.06	1.0	21.3
		3.0	127		3.0	42.7
		5.0	117		5.0	60.0
0.5	0.48	1.0	31.7	0.09	1.0	18.5
		3.0	104		3.0	38.1
		5.0	123		5.0	62.3

[1] mg N/1

[2] cc. acetylene to 70 ml serum bottle with 25 ml water sample.

[3] nM C_2H_4/1-hr.; also equal to nM C_2H_4/mg particulate N-hr. since the water had 1 mg particulate N/1.

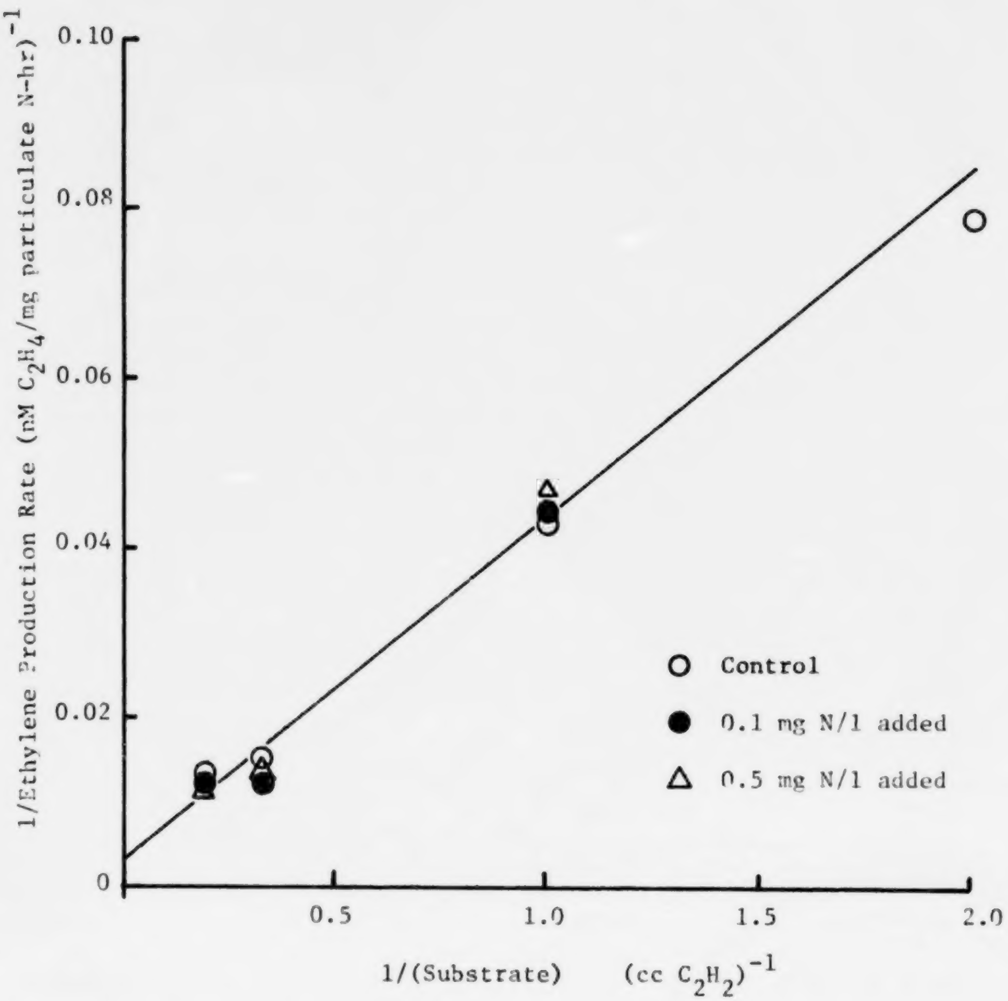


FIGURE 35. ADDITION OF AMMONIA TO NATURAL *ANABAENA* SP. POPULATION HAS NO IMMEDIATE EFFECT ON RATE OF NITROGEN FIXATION.

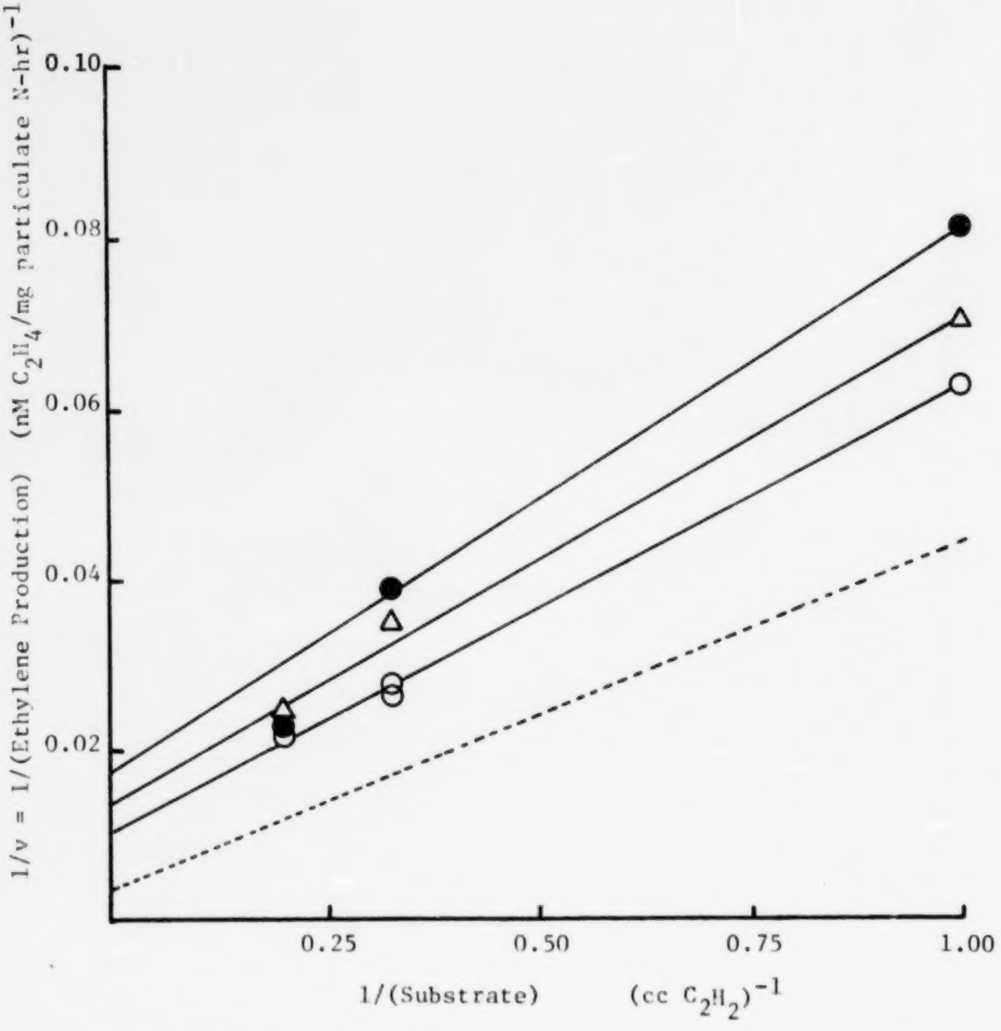
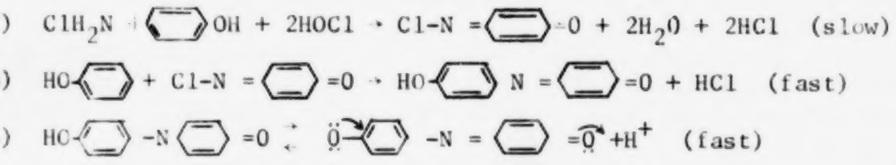


FIGURE 37. EFFECT OF AMMONIA ON NITROGEN FIXATION BY NATURAL ANABAENA SP. POPULATION AFTER INCUBATION WITH ADDED NH_3 FOR 24 HOURS. OPEN CIRCLES, NO AMMONIA ADDED; TRIANGLE, 0.10 MG N/L ADDED; CHOSED CIRCLES, 0.50 MG N/L ADDED. DASHED LINE REPRESENTS INITIAL LINEWEAVER-BURK LINE FOR ALL SAMPLES (SEE FIGURE 36).

tion). Rather the most common automated method for ammonia employs the Berthelot or indophenol reaction in which ammonia, hypochlorite and alkaline phenol react to form a product believed related to indophenol. The product is intensely blue with an absorption maximum around 630 nm. Methods employing this reaction are highly sensitive, but because of its complex chemistry, irreproducible results often are obtained by manual procedures. Bolleter et al. (1961) have proposed the following reaction sequence:

1) $NH_3 + HOCl \rightleftharpoons NH_2Cl + H_2O$ (fast)


2) ClH2N + OH + 2HOCl → Cl-N = =0 + 2H2O + 2HCl (slow)
3) HO + Cl-N = =0 → HO N = =0 + HCl (fast)
4) HO -N =0 ⇌ O -N = =O +H+ (fast)

indophenol blue

But the overall reaction is doubtless more complex than this. Weichselbaum et al. (1969) noted that the reaction has a "very odd behavior" with respect to temperature, order and timing of reagents additions, reagent concentrations, and so forth. They felt that many side and competing reactions (some irreversible) result in a situation where the same equilibrium condition is never reached twice. Many of the problems associated with this method are overcome by use of the Auto Analyzer since all samples and standards are treated identically with respect to order and timing of reagent addition, temperature, reaction time before measurement, etc. With the Auto Analyzer this method essentially becomes a kinetic analysis, i.e. the concentration of ammonia in a sample is determined by the amount reacted after a certain time, not by the amount of product that would be formed at equilibrium. However, because of this fact, the method is particularly susceptible to errors caused by catalysts or inhibitors, which may occur in samples but not in standards, causing variable acceleration or deceleration of reaction rate. Iron, chromium and manganese are known to catalyze the reaction, while oxidants such as persulfate inhibit color development. It has also been reported (Russell, 1944) that certain amino acids respond similarly to ammonia in this procedure.

A less common automated ammonia procedure is the Grasshof method. This sensitive method was developed for sea water analysis (Grasshof, 1966) and is listed as Technicon Auto Analyzer "Industrial Method 42-69W." The chemistry of this procedure is based on the formation of a bromamine intermediate from the reaction of ammonia and bromine. The intermediate reacts with iodide to form iodine, which combines with starch to form the familiar starch-iodine blue complex with an absorption maximum at 570 nm. The method is attractive in certain respects, but it has a highly critical and narrow pH range. This is not an overwhelming problem in seawater analysis since seawater is well buffered within a narrow range, but it is a problem in fresh waters. For example, typical pH values of the 55 Florida lakes ranged from about 4.5 to 9.5 or more (Shannon and Brezonik, 1971c). The Grasshof method was tried in this laboratory for some time, but satisfactory results could not be obtained with Florida waters, and the procedure was abandoned.

Organic color is a common but highly variable constituent of Florida lakes. Many of the oligotrophic lakes in the sand hills are essentially colorless, but in lakes receiving swamp or pine forest runoff color concentrations may reach several hundred units (on the Pt. scale). In fact color levels up to 700 units have been found in Lake Mize. Since the analytical procedures for inorganic nitrogen analysis all involve measurement of color produced by a chemical reaction, it was felt essential to evaluate the influence of naturally occurring organic color on these tests. Empirical evidence indicated at an early date that color was affecting the results of inorganic nitrogen analyses. In particular more problems were encountered with the ammonia analyses than with nitrite and nitrate. Assuming no interaction between organic color and the reagents in the various nitrogen analyses, one would expect the degree of interference from organic color to be greatest for nitrate and least for ammonia since organic color absorbs most strong at the yellow end of the visible spectrum (around 400 nm). The absorption maxima for the nitrate (brucine), nitrite (diazotization) and ammonia (indophenol) procedures are 410,520 and 630 nm., respectively.

To apply compensation methods to these analyses requires careful consideration of the reactions involved. In external compensation, whereby a "key reagent" is removed from the analytical stream to provide a color blank, the "key reagent" must fulfill several criteria. Removing it from the system must not affect the baseline, and since organic color intensity is pH dependent, reagent removal must not alter the reaction mixture pH. Finally, the key reagent must not react with organic color, and its removal obviously must completely eliminate color formation in the reactions involving the analyte. For the most part, selecting reagents which met these criteria was accomplished by trial and error. It was found that sodium hypochlorite was the most suitable reagent to remove in the indophenol procedure for ammonia. Removal of brucine from the mixed reagent of the nitrate procedure was found satisfactory, and in the case of nitrite analysis, removal of the N (1-naphthyl) - ethylenediamine reagent met the established criteria. In order to keep the concentration of the remaining reagents unaltered, deionized water was used to replace the key reagent when color blanks were run.

To determine whether the selected key reagents allowed satisfactory correction of analyses by external compensation (i.e. color blank subtraction), a series of experiments were undertaken with the various analytical procedures. For each analytical procedure a standard or calibration curve was prepared in the usual manner using the range of concentrations normally selected for the particular constituent. A second, similar curve was prepared by adding an identical amount of organic color concentrate to each standard, thus obtaining a series of artificial colored waters having various concentrations of one of the inorganic nitrogen constituents. The color concentrate was obtained by vacuum evaporation at a temperature of about 40°C of colored lake water from Lake Mize in a manner similar to that of Black and Christman (1963). The original sample was concentrated about 50-fold and then filtered through a 0.45 µm Millipore filter and passed through a mixed bed ion exchange resin to remove concentrated inorganic ions. The "purified" concentrate was then stored at $4^{o}C$ in the dark in filled glass containers to minimize oxidation and photodecomposition. The inorganic nitrogen analysis was repeated on each of the color "spiked" samples resulting higher absorbance values for each sample compared to the absorbances of corresponding concentrations in the standard solutions without color. Next, the key reagent was removed from the analytical stream and color blanks were run on each sample. The results for each constituent were plotted on one graph and a curve representing the difference between the absorbance values of color "spiked" standards and their corresponding color blanks was computed and also drawn on each graph.

The results for nitrate, nitrite and ammonia are shown respectively on Figures 38, 29, and 40. In the case of nitrite and nitrate it is apparent that subtraction of the color blank values from the absorbances of the color "spiked" samples results in curves which coincide exactly with the calibration curve using pure standards. This is good evidence that no interaction takes place between the key reagents and organic color, and the results indicate that the external mathod of compensating for effects of organic color can be successfully applied to nitrate and nitrite analyses by these procedures. The validity of this correction technique for nitrate and nitrite assays was verified by attempting recovery of a known amount of standard from solutions of varying color. Solutions of 0.10 mg NO_3^- - N/1 were spiked with color concentrate in levels ranging from 17 to 405 units, and nitrite standards were spiked with color to give final concentrations ranging from 12 to 287 units. The analyses and color blanks were determined, and the latter values subtracted from the former gave the corrected value. Table 30 summarizes the results and shows that application of external compensation yields good agreement with known concentrations.

Results from the ammonia experiment (Figure 40) were at considerable variance with the above data. Subtraction of the color blank curve from the absorbance curve for the spiked standards produced a curve (D) that was still higher than the calibration curve. Further the slopes of the color "spiked" curves were greater than that of the calibration curve, yielding an increasing divergence between them at increasing ammonia levels. Thus, the differences between the calibration curve and the curves for the colored samples cannot be explained solely by assuming that some ammonia was added to the test solutions via the color concentrate. Since all samples received the same amount of color concentrate, the diverging nature of the curves remains unexplained. Interaction of the organic color or of some constituent associated with organic color with the reagents seemed likely. This interaction is further shown by the data shown in Figure 41. These results were obrained by preparing six series of standards solutions. Each series was given a different color, ranging from 0 units for series A to 244 units for series F; within a given series of ammonia standards (ranging from 0 to 1.0 mg N/1) each solution had an equal amount of color. The increasing divergence of the curves as color increased

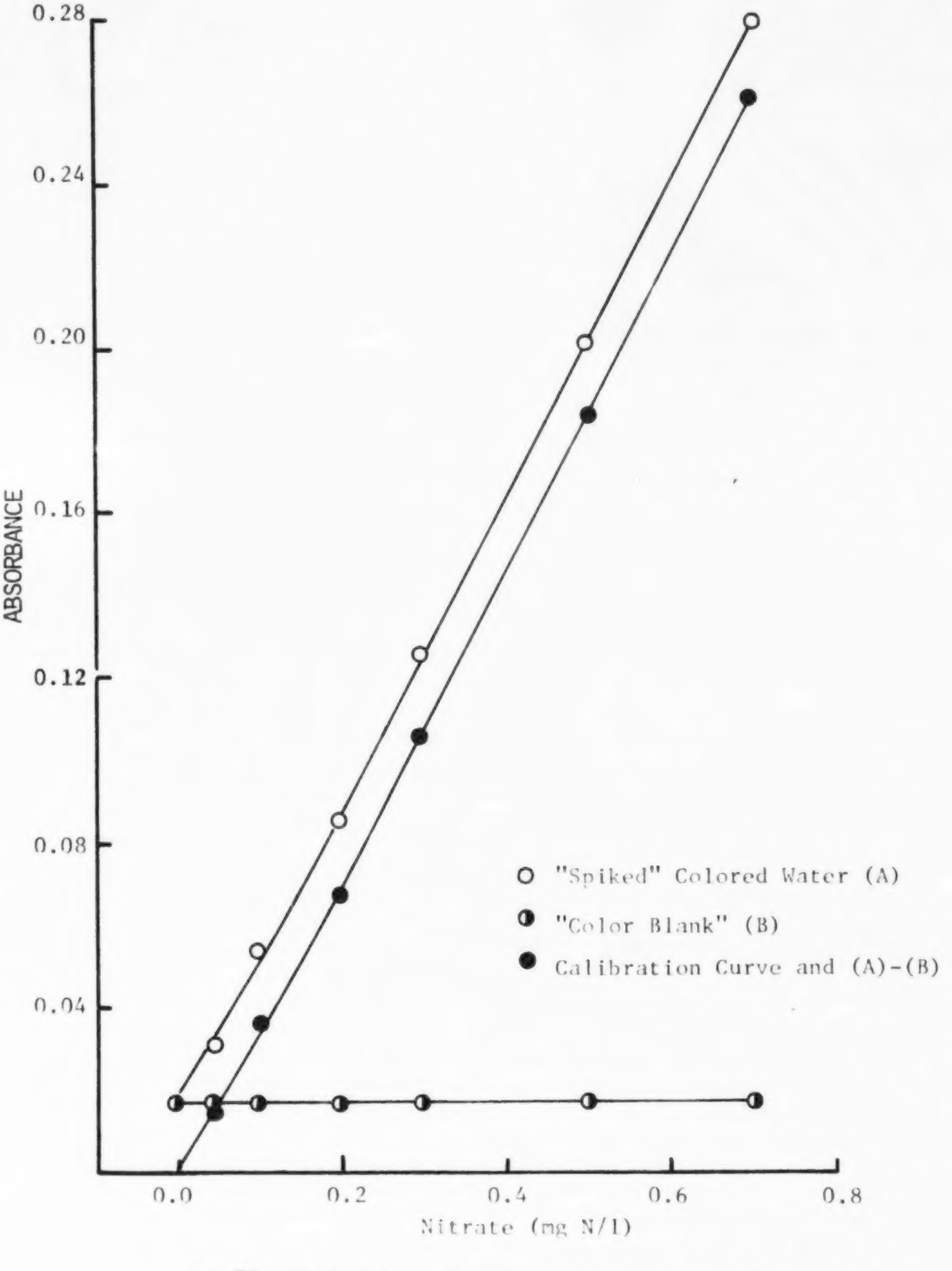


FIGURE 38. EFFECT OF ORGANIC COLOR ON APPARENT NITRATE IN BRUCINE TEST CAN BE ACCURATELY CORRECTED FOR BY METHOD OF BLANK SUBTRACTION. COLOR LEVEL IN TEST SOLUTIONS WAS 63 UNITS (PT SCALE) AT PH 8.3.

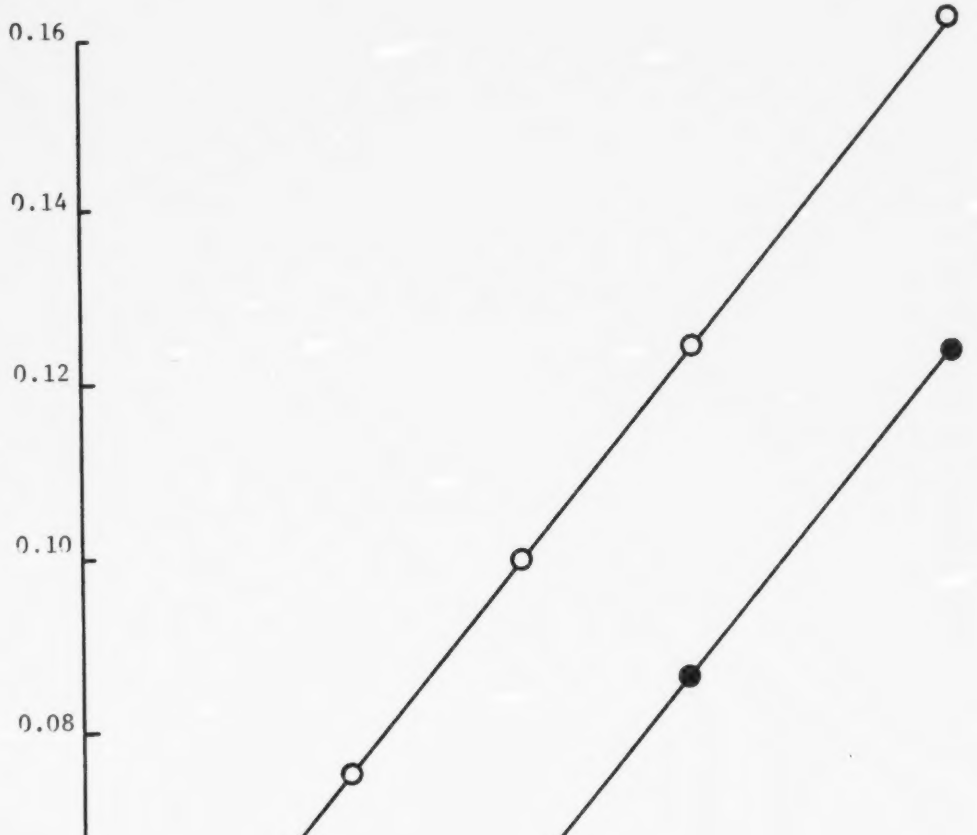


FIGURE 30. EFFECT OF ORGANIC COLOR ON APPARENT NITRITE CONCENTRATION CAN BE ACCURATELY CORRECTED FOR BY METHOD OF BLANK SUBTRACTION. ORGANIC COLOR IN TEST SOLUTIONS WAS 43 UNITS (PT SCALE) AT PH 8.3.

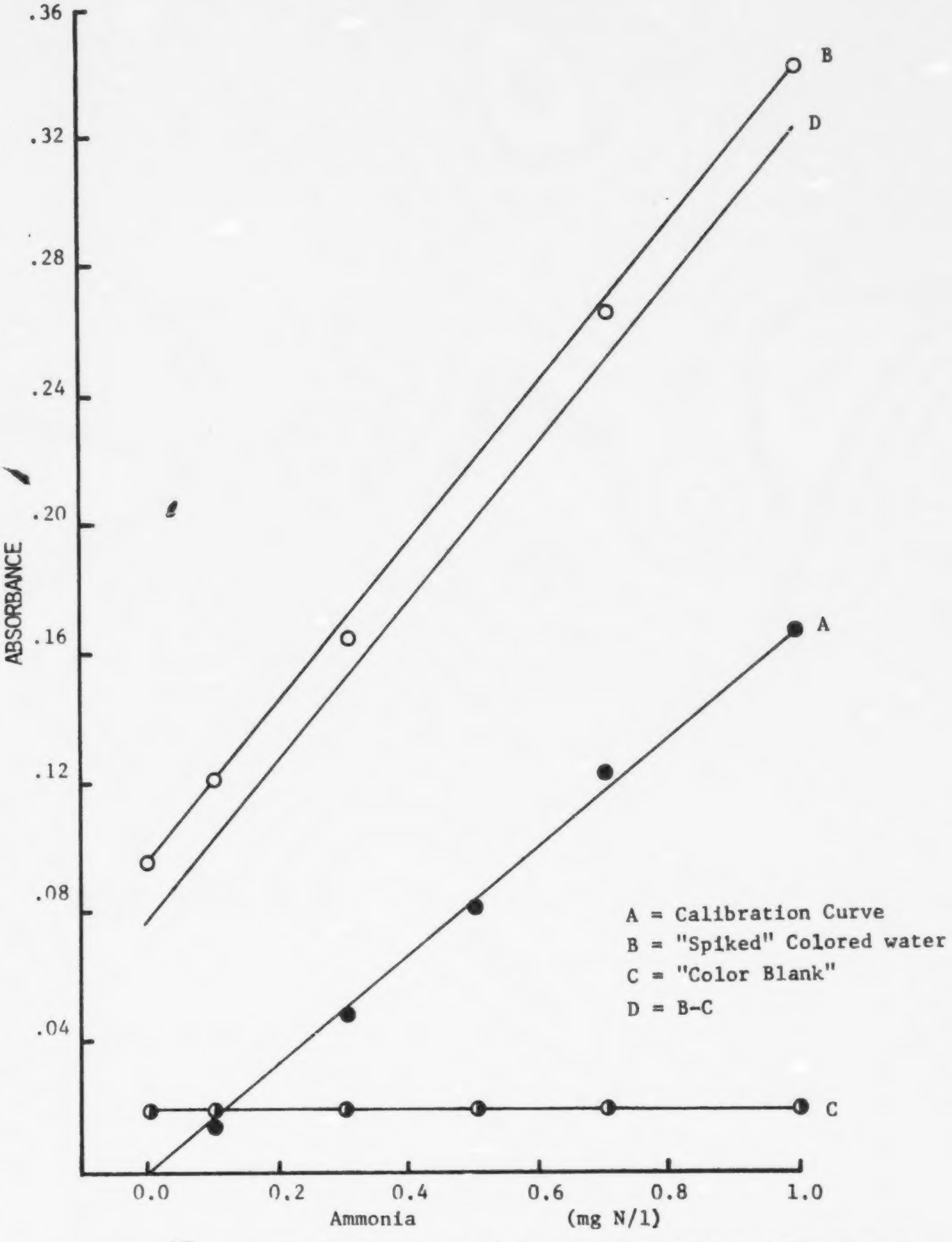


FIGURE 40. EFFECT OF ORGANIC COLOR ON APPARENT AMMONIA CONCENTRATION CANNOT BE ELIMINATED BY BLANK SUBTRACTION. ORGANIC COLOR LEVEL IN TEST SOLUTIONS WAS 105 UNITS (PT SCALE) AT PH 8.3.

Table 30. Application of External Compensation Method to Nitrate and Nitrite Samples Containing Varying Color Concentrations

Synthetic Water	Color	Known	Apparent	Color Blank	Corrected
	Units	NO_3-N, mg N/l	NO_3-N, mg N/l	as NO_3-N, mg N/l	NO_3-N, mg N/l
A	0	0.10	0.10	0.00	0.10
B	17	0.10	0.10	0.02	0.08
C	25	0.10	0.12	0.03	0.09
D	50	0.10	0.15	0.05	0.10
E	84	0.10	0.16	0.07	0.09
F	119	0.10	0.18	0.10	0.08
G	208	0.10	0.25	0.17	0.08
H	405	0.10	0.38	0.30	0.08
		NO_2-N, μg N/l	NO_2-N, μg N/l	as NO_2-N, μg N/l	NO_2-N μg N/l
A	0	5.0	5.0	0.0	5.0
B	12	5.0	5.8	0.8	5.0
C	21	5.0	6.0	0.9	5.1
D	50	5.0	6.9	2.0	4.9
E	130	5.0	9.7	4.8	4.9
F	173	5.0	11.2	6.1	5.1
G	287	5.0	17.2	12.2	5.0

All analytical values are the average of three replicates

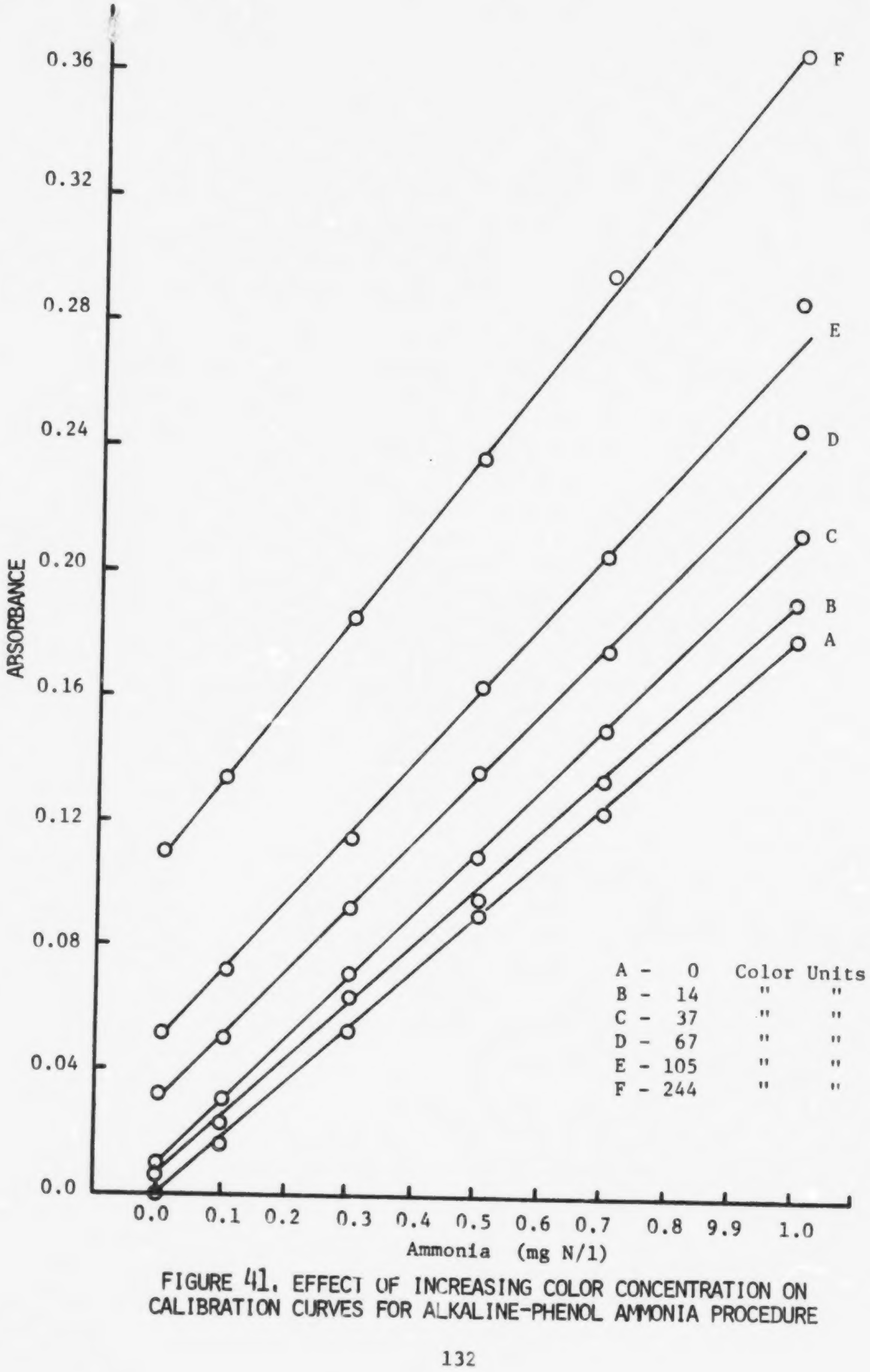


FIGURE 41. EFFECT OF INCREASING COLOR CONCENTRATION ON CALIBRATION CURVES FOR ALKALINE-PHENOL AMMONIA PROCEDURE

supports the supposition that organic color (or some associated constituent) interacts with the reagents in a manner which precludes correction by external compensation. In effect addition of organic color concentrate caused the analytical reaction to proceed toward completion at a rate increasing in proportion to the amount of color plus the concentration of ammonia present.

A possible explanation for these effects derives from consideration of the nature of the analytical reaction (see p. 125) for the indophenol technique and the nature of organic color. The slow or rate determining step of the indophenol reaction involves the reaction of monochloramine with phenol (step 2). Organic color is known to be aromatic in character with many phenolic groups present (Christman and Ghassemi, 1966). Thus, some of these phenolic groups could possibly react with monochloramine under the reaction conditions. In effect, the presence of organic color would increase the concentration of phenol, shift the equilibrium of step 2 to the right, and thus, accelerate the rate determining step. As mentioned earlier, the Auto Analyzer measures the reaction products in this test before the reaction reaches completion. Thus, varying amounts of color would cause various responses to the same ammonia concentration.

If organic color reacted in the manner just described it might be supposed that the formation of extraneous color products (i.e. containing substituted phenols from organic color) would cause a shift in the absorption spectrum of the reaction products. This possibility was studied by measuring the spectra for several reaction mixtures, each containing 0.5 mg NH_3-N/l but with color concentrations from 0 to 378 units (Pt. scale). However, the abosrption maximum for all samples was the same (630 nm.). and the shapes of the spectra were identical (Figure 42). Thus, the possibility of side reactions with phenolic groups from organic color is not substantiated, but it cannot be ruled out solely on this experiment.

An alternate, perhaps simpler explanation for the results with ammonia and organic color is the presence of the catalyst in the color concentrate. It is well known that organic color is frequently associated with high iron levels, and in fact Lake Mize water has a mean iron concentration of 2.4 mg/l (Table 17). Since this iron is associated with the organic color, perhaps as a chelate, or even as an integral part of the color molecule, passing the concentrate through an ion exchange resin would not separate all the iron from the concentrate. As mentioned previously, the indophenol procedure is catalyzed by iron, and in fact some references on the indophenol procedure call for the use of sodium nitroprusside (i.e. sodium pentacyanonitrosyloferrate) to catalyze the reaction, (e.g. see Weichselbaum *et. al.*, 1969). This reagent was not generally used in our analyses since sensitivity was sufficient without it. Further. solutions of this species are unstable and have a relatively short life time, and the appropriate concentration for proper catalysis is fairly critical. That iron associated with organic color is the cause of the problem with the indophenol procedure was indicated by some recent experiments in our laboratory. Addition of the sodium nitroprusside catalyst seems to eliminate the interference due to color (except for the interference due to absorption by color itself, which

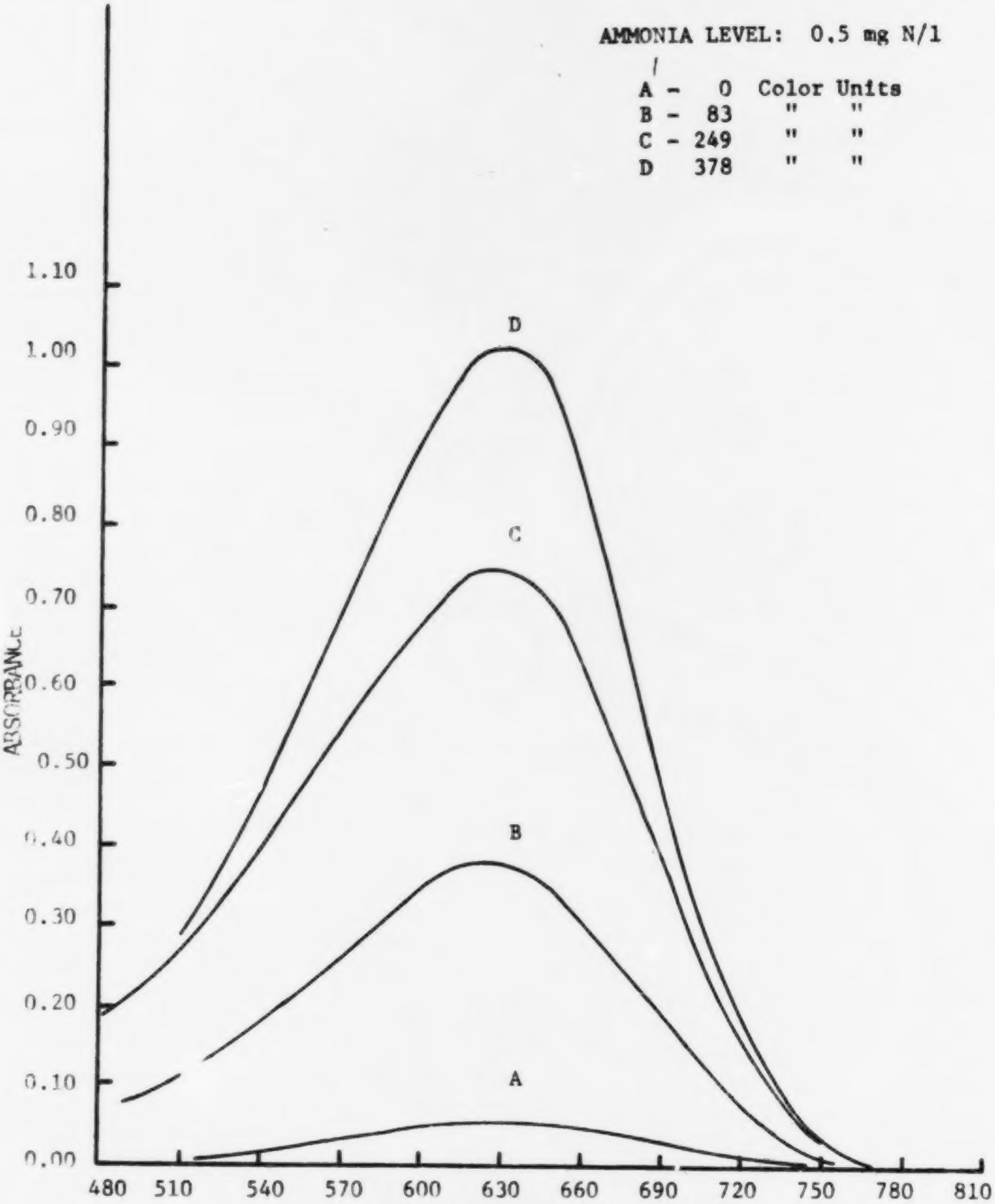


FIGURE 42. ABSORPTION SPECTRA FOR PRODUCTS OF ALKALINE PHENOL-AMMONIA PROCEDURE AT VARIOUS LEVELS OF ORGANIC COLOR

can be corrected by the external compensation method). However, further work will be required to verify this and to determine the optimum level of catalyst to add.

Free amino acids have been known to exist in lake water since the work of Peterson et. al. (1925) on Lake Mendota, Wisconsin. Since then low levels have been reported in English lakes (Fogg and Westlake, 1955) and in marine surface waters (e.g. Degens et. al., 1964). The excretion of free amino acids by zooplankton is a well known phenomenon (Webb and Johannes, 1967), and blue-green algae have also been shown to excrete nitrogenous materials including peptides, amides and amino acids especially in enriched lakes. should not be unexpected. Limited analyses of amino acids in Florida lakes (Yorton, 1971) indicated the presence of low levels (1.5-12 µg/l) of total amino acids, but no definitive work has been undertaken. Because of the chemically similar behavior of amino acids to ammonia in various reactions, a brief study was undertaken to determine whether any amino acids would react in the ammonia procedures employed in our laboratory.

Solutions of 15 common amino acids and urea were prepared so that equivalent N concentrations were approximately 0.5 mg/l in each. Aliquots of each solution were analyzed for ammonia nitrogen by microdistillation followed by Nesslerization, and all were found to be free of ammonia. Aliquots were also digested by micro-Kjeldahl methods to determine the total nitrogen content of each. Each solution was then analyzed by both the indophenol and the Grasshof methods for ammonia nitrogen. The results shown in Table 31 indicate that both methods are susceptible to considerable errors from various amino acids. The Grasshof procedure is much more prone to error than the indophenol method, but all the amino acids tested gave at least some response in both methods. Interestingly urea was not found to react in either method.

In the Grasshof method leucine and methionine give approximately the same response as ammonia. Considering only amino groups as reactive, histidine and argine also fall in this category since only one-third and one-half, respectively, of their nitrogen is in amino groups. The other amino acids gave responses ranging from 23 to 290 percent of equivalent ammonia nitrogen in the Grasshof method. In the indophenol method glycine gave a response equivalent to that of ammonia, and threonine gave a somewhat greater response; the other amino acids gave responses ranging from 4 to 70 percent of the equivalent ammonia nitrogen.

The responses greater than 100 percent can be explained by the fact that the procedures do not measure equilibrium concentrations, but only the extent of reaction after a certain time. The amino acids giving responses greater than 100 percent evidently react more rapidly than ammonia; hence relatively more product has been formed at the time of measurement than is the case for an equivalent ammonia solution. The extent of reaction of non-predictable from the amino acid structure or isoelectric point. The reasons for the relative responses of the various amino acids in these procedures would be an interesting study, but this was beyond the scope of the present project.

Table 31. Response of Automated Ammonia Procedures to Free Amino Acids

Amino Acid	Free NH_3 Present	Total N Present	Grasshoff Method Apparent NH_3-N	 % of Available N	Alkaline-Phenol Method Apparent NH_3-N	 % of Available N
Alanine	<.001	.495	0.60	121	.23	46
Glycine	<.001	.476	1.38	290	.47	99
Arginine	<.001	.405	.18	44	.02	5
Asparagine	<.001	.48	.11	23	.02	4
Aspartic Acid	<.001	.48	.28	58	.06	13
Cysteine	<.001	.33	.10	31	.05	15
Histidine	<.001	.35	.11	31	.03	9
Leucine	<.001	.495	.53	107	.07	14
Lysine	<.001	.374	.74	198	.05	13
Methionine	<.001	.47	.49	104	.31	66
Phenylalanine	<.001	.462	.52	113	.15	32
Proline	<.001	.445	1.03	231	.17	38
Serine	<.001	.478	.59	122	.34	70
Threonine	<.001	.495	.25	51	.60	121
Valine	<.001	.47	.60	128	.04	9
Urea	<.001	.445	.08	18	00	00

All values in mg N/liter.

The need for better automated procedures for ammonia should be apparent from the studies reported above. The interference resulting from color or color correlated species is significant and not correctable by usual compensation methods. The source of the interference is still not known with certainty, but iron is the most attractive candidate. If this is substantiated, at least this error can be eliminated by swamping out the natural iron in the sample with added catalyst (sodium nitroprusside). However, the interferences resulting from amino acids may be more serious. The normal levels of free amino acids in natural waters, compared to the levels of ammonia are not well known, but it should be noted that ammonia concentrations in the μg N/l range are of great interest in limiting nutrient studies. Limited information suggests that free amino acids occur in waters in these concentrations, and the introduction of serious errors in low level ammonia analyses is thus a distinct possibility.

SECTION X

ACKNOWLEDGEMENTS

The able assistance of Michael A. Keirn and Roger A. Yorton in conducting the research culminating in this report is gratefully acknowledged. Mr. Keirn conducted or directed most of the nitrogen fixation studies and was responsible for the bacterial isolations. Mr. Yorton performed or directed the various chemical analyses throughout the project.

Carol C. Harper worked on initial phases of the nitrogen fixation studies, and Roger King assisted in various aspects of sampling and analysis. Glenn T. Brasington was most cooperative in performing sampling and other duties especially during diurnal and other extended studies. The assistance of W. H. Morgan in fiscal matters greatly simplified administration of this grant. The principal investigator was Patrick L. Brezonik, who directed the project and wrote this report.

The support of the project by the Office of Research and Monitoring, Environmental Protection Agency, and the cooperation and assistance of C. Powers, grant project officer, are acknowledged with sincere thanks.

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SECTION XI

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SECTION XII

PUBLICATIONS AND PATENTS

The following publications have resulted from work supported wholly or in part by this project:

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Brooks, R. H., P. L. Brezonik, H. D. Putnam, and M. A. Keirn. 1971. Nitrogen fixation in an estuarine environment: The Waccasassa on the Florida Gulf coast. Limnol. Oceanogr. 16 (5), 701-710.

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Yorton, R. A. and P. L. Brezonik. Interferences in the analyses of inorganic nitrogen and phosphate in natural waters and procedures for correction. Manuscript in preparation for submission to J. Am. Wat. Works Assoc.

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SECTION XIII

APPENDIX A

SAMPLING AND ANALYTICAL METHODS

1. Sampling Procedures.

Water samples for routine chemistry were taken by Van Dorn sampler; bacteriological samples from the depths were taken aseptically in BOD bottles attached to a special apparatus which opens the bottle from the surface at the pull of a string. Sediment cores 30 to 50 cm. in length were collected in plexiglass tubes. Surface sediments were collected with an Ekman dredge.

2. Routine Chemical Analyses.

Acidity, alkalinity, and pH were run potentiometrically in the laborataory within a few hours after collection of samples in BOD bottles to avoid contact with air. Dissolved oxygen was determined by the Winkler-azide method (A.P.H.A. 1965). Cations were measured by atomic absorption spectrophotometry. Other determinations were done according to Standard Methods (A.P.H.A. 1965).

3. Nutrient Analyses.

Inorganic nitrogen and phosphorus forms were determined with a Technicon Auto Analyzer on samples preserved with one ml saturated mercuric chloride per liter of sample. Ammonia was determined using modifications (Yorton, 1971) of the phenol-hypochlorite method (Technicon Corp., 1969). Nitrate was determined by the automated brucine technique (Kahn and Brezenski, 1967), and nitrite by adaption of the *Standard Methods* diazotization procedure to the Auto Analyzer. Ortho phosphate was measured by the Murphy and Riley (1962) single reagent method. Flow diagrams and details of the automated version of these methods are presented by Yorton (1971). Total organic nitrogen was determined by the macro-Kjeldahl method according to *Standard Methods*; recovered ammonia was measured by titration or by the Auto Analyzer. Particulate organic nitrogen was measured by micro or macro-Kjeldahl on solids scraped from an $MgCO_3$ mat placed on a 0.45 μm. Millipore filter. Total phosphate analyses were performed by persulfate-sulfuric acid autoclaving for one hour at 15 psi followed by manual determination of ortho phosphate by the Murphy and Riley (1962) method.

4. Biological Methods.

The acetylene reduction technique (Stewart *et al.*, 1967) was used to measure rates of nitrogen fixation. The technique used on water

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samples was described by Brezonik and Harper (1969) and on sediments by Brooks et al. (1971). Sediment and water samples (20 to 25 ml) were placed in 70 ml serum bottles, capped and purged with helium or, in the case of waters containing dissolved oxygen, with a gas mixture (20% O_2, 0.03% CO_2, balance Ar). Following this 5 cc. of gas phase was removed with a gas tight syringe and replaced with 5 cc. purified acetylene (Matheson Co.). After incubation (usually for 1-2 hours) activity was stopped by adding 1 cc. 50% trichloroacetic acid with a needle and syringe. Gas separation and ethylene analysis was accomplished with a Varian-Aerograph 600 D gas chromatograph with a hydrogen flame ionization detector and a 1/8" x 6' Poropak R column at room temperature. Newnan's Lake and Bivin's Arm samples were incubated in situ. Other lake and sediment samples were brought to the laboratory for processing and incubation at 22°C in a water bath-shaker. Sediments and anoxic water samples were incubated in the dark; aerobic samples were incubated under daylight-type fluorescent lighting. Controls run with each set of samples were carried through the identical procedure except that 1 ml 50% TCA was added before acetylene addition.

Primary production was assayed by ^{14}C techniques with in situ incubation for 1 to 3 hours and Geiger counting of the ^{14}C incorporated into particulate matter (filtered onto 0.45 μm. Millipore filters). Chlorophyll a was measured by standard acetone extraction and spectrophotometry (Creitz and Richards, 1955) using Parsons and Strickland's (1963) equations to calculate chlorophyll concentration.

5. Sediment Analyses.

Volatile solids were determined by Standard Methods (A.P.H.A., 1965). Total organic nitrogen and free ammonia determinations were made on fresh-sediments diluted with demineralized water into a thin slurry. Ammonia was distilled from the slurry buffered at pH 8.3 with sodium bicarbonate and the recovered ammonia measured by titration. Total organic nitrogen was determined similarly by micro-Kjeldahl digestion. Results were converted to a dry weight basis by drying and weighing a known volume of the sediment slurry. Elemental analyses were accomplished with a Perkin-Elmer C-H-N analyzer in oven (60°C) dried sediment samples which were pulverized to a homogenous mixture in a mortar and pestle. Total phosphate was run on fresh sediment slurries using persulfate oxidation and the Murphy and Riley (1962) manual method for ortho phosphate.

References cited in this appendix are listed in Section X.

Appendix B

Routine Data Collected on Newnan's Lake and Bivin's Arm During Nitrogen Fixation Study

Bivin's Arm

Parameter[1]	21 May	6 June	25 June	9 July	29 July	7 Aug	21 Aug	3 Sept	18 Sept	2 Oct	16 Oct	30 Oct	13 Nov
Air Temp.	29.0	25.7	32.0	--	36.0	38.4	27.6	28.6	33.9	25.3	32.0	20.5	16.4
Surf. water temp.	27.3	28.2	31.1	--	31.5	31.3	27.6	27.5	27.2	25.9	29.0	20.5	17.8
Cloud cover	20	100	0	90	80	80	5	90	30	100	5	100	5
Wind	0-2	5	0-2	0	5	0	10	5	5	5	0	10-15	5
Secchi Disc.	0.6	0.3	0.45	0.3	0.3	0.6	0.6	0.75	0.45	0.9	0.45	0.45	0.45
Dis. O_2													
surface	10.5	13.0	12.1	10.5	12.3	13.3	12.6	3.8	9.9	4.6	15.0	10.0	10.8
bottom	8.4	12.5	12.0	10.0	10.6	9.4	12.1	3.7	9.8	4.1	14.4	9.4	11.0
pH	8.9	9.5	9.2	9.5	9.9	9.5	9.5	8.7	9.8	8.8	9.6	9.2	8.9
Alkalinity as $CaCO_3$	97	88	95	113	115	145	100	100	125	96	105	155	160
Tot. org. N	2.7	3.3	4.0	5.4	2.9	2.30	3.0	1.7	3.25	1.1	2.4	2.6	2.4
NH_3-N	0.70	0.11	0.80	0.0	--	0.21	0.48	0.76	0.62	0.38	0.35	0.73	0.42
NO_2^--N	0.008	0.003	0.001	0.001	--	--	0.000	0.003	--	0.006	0.000	0.004	0.006
NO_3^--N	0.03	0.00	0.00	0.00	--	0.01	0.00	0.04	0.03	0.21	0.00	0.06	0.00
Ortho PO_4-P	0.5	0.042	0.063	0.091	--	0.3	0.25	0.27	0.23	0.19	0.13	0.30	0.02
Total PO_4-P	1.0	0.37	0.56	0.58	--	0.61	0.49	0.49	0.72	0.54	0.54	0.58	0.51
Prim prod.	256	1070	790	2760	1480	117	1960	100	1580	166	1220	420	81
N_2fixed (a)	0	7.2	114	25	46	109	330	46	0	0	42	0	0
(b)	0	0.067	1.08	0.24	0.43	1.02	3.06	0.43	0	0	0.39	0	0

Bivin's Arm

Parameter[1]	24 Nov	11 Dec	2 Jan	12 Jan	21 Jan	4 Feb	18 Feb	9 Mar	31 Mar	7 Apr	28 Apr	12 May	26 May
Air temp.	23.3	16.3	10.5	10	8.5	10	15.5	21	24.5	22.7	27.5	26.8	28.8
Surf. water temp.	15.8	15.3	12.8	9.3	12.9	13.0	16.1	17.8	24.5	21.9	26.8	25.3	24.8
Cloud cover	10	15	100	100	0	0	95	15	30	5	75	50	10
Wind	0-3	5-10	10	5	5	5	0-5	5	10	5	0	0-5	5
Secchi disc.	0.45	0.3	0.45	0.85	0.75	0.45	0.6	0.45	0.75	0.75	0.55	0.45	0.55
Dis. O_2 surf.	11.4	8.8	8.4	10.2	9.5	8.8	11.8	6.9	8.5	7.9	12.2	9.9	7.5
bottom	11.4	8.6	8.1	10.8	9.4	8.7	10.9	6.7	6.5	8.6	8.7	8.2	7.5
pH	8.8	8.4	9.0	8.0	7.9	8.3	8.9	8.4	8.4	8.0	8.9	9.0	8.8
Alkalinity	--	110	100	110	120	80	100	115	98	105	120	185	110
Tot. org. N	1.8	1.8	2.0	1.0	1.8	1.2	1.7	1.8	1.0	1.1	1.7	2.1	1.6
NH_3-N	0.00	0.07	0.23	0.16	1.0	0.08	0.00	0.20	0.22	0.34	0.13	0.55	0.56
NO_2^--N	0.000	0.001	0.000	0.036	0.045	0.000	0.005	0.006	0.002	0.006	0.000	0.000	0.000
NO_3^--N	0.00	0.00	0.03	0.14	0.67	0.14	0.03	0.02	0.03	0.03	0.02	0.01	0.00
Ortho PO_4-P	0.15	0.16	0.055	0.30	0.33	0.031	0.43	0.48	0.40	0.23	0.43	0.21	0.13
Total PO_4-P	0.41	0.51	0.21	0.54	0.50	0.63	0.62	0.70	0.62	0.73	0.59	0.65	0.52
Prim. prod.	47	324	236	84	650	168	25	68	263	81	345	400	585
N_2 fixed (a)	0	12.9	15.7	0	13.4	0	17.7	0	0	1.8	3.6	0	0
(b)	0	.12	.15	0	.12	0	.17	0	0	.016	.034	0	0

[1] Temperature °C; cloud cover in percent; wind in mph; Secchi disc in m.; all concentrations in mg/l of species noted; primary production in mg C/m^3-hr.; nitrogen fixation (a) in nM ethylene/m^3-hr; and nitrogen fixation (b) in ng N/l-hr. assuming a theoreticl molar ratio of 1.5 moles ethylene produced per mole of ammonia.

Newnan's Lake

1969

Parameter[1]	20 May	3 June	24 June	8 July	24 July	6 Aug	22 Aug	9 Sept	17 Sept	25 Sept	14 Oct	23 Oct	7 Nov
Air temp.	26.6	26.0	32.7	31.2	32.5	36.4	31.1	24.1	37.1	26.8	32.0	23.1	20.9
Water temp.													
surf.	28.0	27.0	31.7	31.5	30.0	29.1	30.8	28.1	32.3	27.5	28.5	24.6	16.9
bottom	25.1	27.0	28.9	31.0	29.7	27.9	30.5	28.0	26.5	27.0	26.0	23.8	16.4
Cloud cover	--	0	40	20	80	20	30	90-100	50	20	40	45	60
Wind	--	--	0	5-10	10	5	--	5-10	0	--	--	10	5
Secchi Disc.	0.6	0.4	0.45	0.3	0.45	0.6	0.45	0.45	1.05	0.6	0.6	0.6	0.45
Dis O_2 surf.	8.6	8.3	8.7	7.7	8.0	9.1	7.9	8.0	7.8	6.2	8.0	8.0	9.4
bottom	8.1	8.0	8.0	7.6	8.3	6.5	7.9	7.9	7.2	6.2	6.3	7.9	8.6
pH	8.8	7.1	8.6	7.7	8.0	9.1	7.5	7.4	7.1	6.6	7.2	7.1	6.9
Alkalinity as $CaCO_3$	8.4	2.9	6.8	10	12.5	8.5	7.5	7.2	7.2	7.2	7.7	8.6	7.2
Acidity as $CaCO_3$	0	1.4	0	0	0	0	--	--	1.9	3.0	2.1	1.7	2.5
Tot. org. N	1.9	1.8	1.5	1.7	2.1	1.2	1.8	1.5	1.3	1.3	1.3	2.3	1.4
NH_3-N	0.69	0.80	0.07	0.07	--	0.03	0.18	0.04	0.02	0.10	0.29	0.32	0.48
NO_2^--N	.006	.006	.001	.002	--	--	.000	.000	.000	.000	.001	.003	.003
NO_3^--N	0.16	0.22	0.00	0.00	--	0.02	0.03	0.11	0.00	0.01	0.02	0.06	0.16
Ortho PO_4-P	0.012	0.028	0.015	0.09	--	0.009	0.010	0.004	0.000	0.000	0.003	0.010	0.012
Total PO_4-P	0.16	0.15	0.15	0.25	--	0.10	0.14	0.09	0.15	0.14	0.08	0.12	0.10
Prim. prod.	223	15	169	229	331	247	67	23	54	86	63	48	39
N_2 fixed (a)	0	0	0	0	76.5	0	0	0	0	0	21	0	0
(b)	0	0	0	0	0.71	0	0	0	0	0	0.02	0	0

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[1] See footnote at end of table for parameter units.

Newnan's Lake

Parameter[1]	20 Nov 1969	4 Dec 1969	8 Dec 1969	11 Dec 1969	16 Dec 1969	18 Dec 1969	23 Dec 1969	26 Dec 1969	30 Dec 1969	2 Jan 1970	7 Jan 1970	13 Jan 1970	20 Jan 1970
Air temp.	26.3	15.7	19.3	14.4	15.7	18.5	13.2	13.0	22.0	10.6	9.0	7.0	11.9
Water temp. surf.	14.3	13.4	14.5	15.0	13.7	13.9	13.0	13.8	14.6	13.6	12.5	8.2	12.8
" " bottom	14.0	13.1	13.4	15.0	13.5	12.5	12.6	13.8	14.4	13.6	12.5	8.1	12.5
Cloud cover	100	30	5	25	40	20	10	0	60	100	75	100	75
Light intensity	--	--	--	6080	5000	6400	6400	7040	4160	510	2720	640	720
Wind	10	10	0-5	5	5	0-5	0	15	10	10	20	10	0
Secchi disc.	0.55	0.45	0.4	0.25	0.45	0.5	0.45	0.4	0.25	0.25	0.3	0.4	0.4
Dis. O_2 surf.	9.9	9.3	11.1	9.7	11.9	--	11.4	10.4	--	10.1	10.1	11.4	12.4
" " bottom	10.0	10.5	--	--	--	--	--	--	--	--	--	--	--
pH	7.1	7.8	8.4	7.0	8.5	--	7.8	7.0	--	6.8	6.7	6.6	6.9
Alkalinity as $CaCO_3$	8.2	8.7	8.9	8.7	8.6	--	--	12	--	1.2	1.5	6.1	0
Acidity as $CaCO_3$	1.6	0.6	0	2.3	0	--	--	2	2.2	2.8	4.6	3.2	2.2
Tot. org. N	1.7	2.4	2.0	2.1	3.9	1.6	2.0	2.1	2.2	0.7	0.7	1.8	2.2
NH_3-N	0.33	0.60	0.31	0.06	0.23	0.09	0.19	0.19	0.18	0.20	0.02	0.10	--
NO_2^--N	0.002	--	--	0.000	0.000	0.001	--	--	--	--	0.000	0.003	0.000
NO_3^--N	0.05	0.09	0.02	0.06	0.02	0.05	0.14	0.14	0.05	0.07	0.05	0.05	0.04
Ortho PO_4-P	0.007	0.010	0.004	0.007	0.009	0.007	0.013	0.005	0.012	0.005	0.005	0.007	0.013
Total PO_4-P	0.15	0.12	0.17	0.14	0.14	0.05	0.15	0.19	0.19	0.15	0.21	0.14	0.050
Chlorophyll a	--	--	66.6	67.9	78	10.4	--	10.4	10.9	10.0	16.5	73	60
Prim. production	36	70	34	119	41	160	77	11.2	35	4.8	15.5	43	32
N fixation (a)	0	41	24.5	20.3	28.6	31.2	11.4	15.2	22.1	5.7	6.5	2.7	0.5
(b)	0	0.38	0.23	0.19	0.27	0.29	0.11	0.14	0.21	0.053	0.06	0.025	0.004

[1] See footnote at end of table for parameter units.

Cont'd.

Newnan's Lake

1970

Parameter [1]	30 Jan	3 Feb	16 Feb	27 Feb	4 Mar	11 Mar	20 Mar	26 Mar	2 Apr	7 Apr	24 Apr	6 May	22 May	2 June
Air Temp.	11.0	12.5	18.9	16.4	19.2	22.4	28.5	20.1	23.0	15.2	30.4	27.1	31.8	32.1
Water temp. surf.	13.7	14.2	13.9	16.4	17.5	19.0	18.3	18.5	22.5	19.1	27.0	26.5	27.0	28.2
bottom	13.4	14.2	12.1	13.0	16.3	18.5	17.2	18.2	22.5	19.0	25.4	25.5	26.0	28.0
Cloud cover	70	100	100	0	60	40	50	100	100	0	95	50	40	100
Light intensity	2880	1030	1020	3860	4970	8000	800	1280	--	--	6400	7000	6400	5000
Wind	15	15-20	5		5	10	10	0	25	5	--	5	15	5
Secchi Disc.	0.45	0.45	0.45	0.7	0.75	0.8	0.8	0.75	.35	0.6	0.5	1.0	0.5	0.8
Dis O_2 surf.	12.6	8.5	10.5	8.9	9.9	8.0	10.2	10.2	7.5	7.5	7.0	8.6	8.8	8.2
bottom	--	--	--	--	--	--	--	--	7.5	6.7	6.8	8.0	8.4	8.0
pH	6.8	7.0	7.1	6.7	7.3	7.4	6.7	7.2	6.9	6.8	6.8	7.2	7.5	7.0
Alkalinity	0	1.6	5.8	8.0	12.7	16.0	0.4	6.3	4.2	6.5	3.5	10	6.4	6.3
Acidity as $CaCO_3$	2.7	2.1	1.2	2.9	3.2	4.0	2.9	0.9	1.8	2.2	1.9	2.5	0.6	2.1
Tot. org. N	1.7	1.1	0.7	1.0	1.4	1.1	1.2	1.5	1.3	1.2	1.2	1.1	1.9	2.0
NH_3-N	0.23	0.10	0.06	0.21	0.15	0.33	0.06	0.08	0.28	0.40	0.79	0.0	0.25	0.36
NO_2^--N	.000	.000	.000	.001	.000	.006	.002	.000	.003	.003	.008	.000	.001	.003
NO_3^--N	0.07	0.05	0.02	0.05	0.05	0.02	0.11	0.05	0.03	0.04	0.03	0.03	0.08	0.02
Ortho PO_4-P	0.007	0.090	0.008	0.011	0.006	0.012	0.007	0.001	0.010	0.017	0.042	0.008	0.011	0.011
Total PO_4-P	0.060	0.11	0.09	0.09	--	0.08	0.12	0.12	0.15	0.13	0.064	0.12	0.056	0.063
Chlorophyll *a*	57	67	49	78	58	62	51	68	55	87	68	43	80	101
Prim. production	--	29	--	85	117	281	281	270	22	197	29	129	185	65
N_2 fixation (a)	2.8	--	14.3	17.9	23	39	27.6	11.8	0	3.2	1.5	1.6	0	0
(b)	0.025	--	0.13	0.17	0.21	0.36	0.26	0.11	0	0.03	0.014	0.015	0	0

[1] Temperature in °C; cloud cover in percent; wind in mph; Secchi disc in meters; chemical concentrations in mg/l of species noted; chlorophyll a in mg/m^3 primary production in mg C/m^3-hr.; nitrogen fixation (a) in nM C_2H_4/m^3-hr.; and nitrogen fixation (b) in ng N/l-hr. assuming a theoretical producation ratio of 1.5 moles ethylene produced per mole of ammonia fixed.

APPENDIX C

ENRICHMENT AND ISOLATION PROCEDURES FOR NITROGEN FIXING AGENTS IN LAKE WATER AND SEDIMENTS

Water samples were collected from various depths in Lake Mize aseptically by lowering a closed, autoclaved BOD bottle to the appropriate depth and opening the stopper with a string. Lake water samples were subjected to enrichment schemes for heterotrophic anaerobic, facultative, or aerobic bacteria, photosynthetic bacteria, and yeasts and fungi. In addition, samples were examined microscopically for blue-green algae and photosynthetic bacteria but visual examination of all samples yielded negative results.

Heterotrophic nitrogen fixing bacteria, both aerobic and anaerobic, were enriched intially using a nitrogen free, modified Winogradsky's medium (Grau and Wilson, 1962) with sucrose as the carbon source. Aliquots from each depth were mixed with equal amounts of double strength medium and incubated at 20°C both aerobically and anaerobically under one atm N_2. Control aliquots were carried through the same procedure with the exception that 1.0 g/l NH_4Cl was present in this medium. After growth was noted by increased turbidity, transfers were made to fresh nitrogen free media. After three transfers streak plates were made on to the same medium plus 1.5% agar.

Enrichment for photosynthetic bacteria was done with three media. Athiorhodaceae (non-sulfur purple bacteria) were enriched by placing 100 ml of lake water from each depth into A BOD bottle and filling with a medium consisting of sodium acetate (2 g/l), NH_4CL (1 g/l), K_2HPO_4 (0.5 g/l), $MgCl_2$ (0.1 g/l), yeast extract (0.05 g/l), and adjusted to pH 7.0. The bottles were stoppered and incubated at 25°C under 250 foot candles of continuous fluorescent light. Enrichment for Thiorhodaceae (green and purple sulfur bacteria) was accomplished by inoculating 200 ml of lake water into each of two BOD bottles containing 100 ml of a sterile slurry of cellulose (3 g.), $CaSO_4$ (3 g.), NH_4Cl (0.139 g.), KH_2PO_4 (0.33 g.), and $Ha_2S \cdot 7H_2O$ (0.07 g.), one adjusted to pH 7.3 (for green sulfur bacteria) and the other adjusted to pH 8.5 (for purple sulfur bacteria). The bottles were capped and incubated as described above for the Athiorhodaceae. Controls, consisting of sediment samples from a lake known to contain these groups (Lake Alice, Gainesville, Florida (Lackey *et al.*, 1964), were carried through the procedure to assure that the scheme was valid.

Enrichment for nitrogen fixing yeasts of fungi was made by filtering aliquots of water through 0.45 μm. membrane filters which were incubated at 25°C and 35°C on Sabouraud dextrose agar. All morphological types (about 30% of the total colonies or 65 isolates) were picked after 4 days growth, gram stained and inoculated into a nitrogen deficient medium modified from Metcalfe and Brown (1957), containing 0.3% sodium benzoate; glucose, sucrose, and mannitol glucose as carbon sources at 5.0 g/l; phosphate, and trace metals and buffered to pH 7.2. The isolates were also inoculated into a control medium (i.e. the above medium plus nitrogen as NH_4Cl). All cultures were incubated in the dark at 25°C.

Anaerobic bacteria such as occur in the genus Clostridium were considered the most likely organisms responsible for fixation in the anoxic estuarine sediments, and the following enrichment and isolation scheme for clostridia-like nitrogen-fixing organisms was used to determine whether such organisms occur in Waccasassa Estuary sediments. A sediment sample from the 2-5 cm. depth was heated at 80°C for 10 minutes to destroy vegetative forms and then inoculated into a nitrogen-free salt medium containing 4 g/l of one of the following carbon sources: acetate, maltose, mannitol, sorbitol or sucrose. All of these except acetate have been reported as fermentable carbon sources for Clostridium pasteurianum. After incubation at room temperature (22-25°C) under a pure nitrogen atmosphere for three days, growth was apparent (by increased turbidity and gas production) in all samples except the medium containing acetate as carbon source. Most rapid growth occurred initially with mannitol; however in subsequent transfers sorbitol gave quickest response. A test for acetylene reduction activity in the mannitol culture gave positive results. Gram studies showed an abundance of gram-positive rods accompanied by a few gram-variable coccoid forms. Many of the rods showed sub-terminal swellings characteristic of developing spores.

The enrichment cultures were incubated five more days to promote sporulation, then heated to 80°C for 10 minutes and transferred to basal salts media containing sorbitol. Transfers were incubated under an N_2 atmosphere at room temperature for 48 hours, and another transfer was made to similar media. Following incubation for 5 days, a third transfer was made. Micro-Kjeldahl analysis of each batch of fresh media showed no detectable ammonia or organic N. A test for acetylene reduction on the 8 bottles (duplicates of the 4 original carbon sources showing growth) from the third transfer gave positive results for 3 bottles.

Streak plates were made onto the nitrogen-free basal salts medium plus sorbitol in 2 percent agar from the incubated material of the third transfer, and the plates were incubated for 72 hours in an anerobic jar (N_2 atmosphere). Colonies on the plates were all similar in appearance and did not display chromogenesis. Ten colonies which were gram-positive rods were picked from plates streaked with media from bottles which exhibited acetylene reduction and were transferred to basal salts plus sorbitol. Each isolate was grown under both aerobic and anaerobic (N_2 atmosphere) conditions. Four colonies grew only anaerobically; the remainder grew under both conditions. The latter were tested for acetylene reduction and gave negative results. The four obligate anaerobic isolates were also tested for acetylene reduction, and three of the isolates showed positive rates.

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